MOLECULAR AND PHEROMONE STUDIES OF PECAN NUT CASEBEARER, *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE)

A Thesis

by

EMILIE ANNE HARTFIELD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Entomology

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Approved by:

Chair of Committee,	Raul F. Medina
Committee Members,	Marvin K. Harris
	Adam G. Jones
Head of Department,	Kevin M. Heinz

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ABSTRACT

Molecular and Pheromone Studies of Pecan Nut Casebearer, *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae). (December 2009)

Emilie Anne Hartfield, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Raul F. Medina

The pecan nut casebearer, *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae) is the most damaging insect pest of pecan, *Carya illinoinensis* (Wang) K. Koch (Fagales: Juglandaceae). Two sex pheromones have been identified for this species and are currently being used to assist pecan growers in the timing of insecticide applications. The discovery that there are two pheromone types produced by *A. nuxvorella* has led to complications in the implementation of pheromone monitoring programs. One pheromone (referred to as standard) is attractive to moths in the southern US, but not in Mexico. The other pheromone (referred to as Mexican) is attractive to moths in the southern US and in Mexico. Because most male lepidopterans respond only to a specific pheromone, it was suspected that there were two pheromone strains of *A. nuxvorella*, one exclusively present in the northern distribution of *A. nuxvorella* (US strain) and the other widely distributed from Sonora, Chihuahua, and Durango in Northern Mexico to Texas, Georgia, and Oklahoma in the US (Mexican strain).

In order to confirm the existence of the two alleged pheromone strains, AFLP markers were obtained and analyzed, male response to pheromones was observed and

phenological differences were assessed. Additionally, the relative abundance of each of the two pherotypes was evaluated and the population structure of this pest across its geographic distribution was determined.

Results of genetic analysis show that the genetic differentiation between these insects is not explained by pheromone type. This information is further supported by a pheromone assay in which a large proportion of US collected *A. nuxvorella* males and Mexican collected *A. nuxvorella* males chose both pheromones when tested multiple times. Furthermore, no phenological differences were detected between the two pherotypes in the US, although significantly more male *A. nuxvorella* in the US are attracted to field-deployed pheromone traps baited with the standard pheromone than the Mexican pheromone. Finally, population genetic analyses indicate a high degree of genetic structure in *A. nuxvorella* across its geographic distribution, with the genetically distinct populations occurring in areas where *A. nuxvorella* is not native, but has been introduced.

DEDICATION

To my family

ACKNOWLEDGEMENTS

I would like to thank all of my committee members, Dr. Raul F. Medina, Dr. Marvin K. Harris, and Dr. Adam G. Jones for their guidance and support. I would like to thank all of the members of the Medina Lab for their assistance. I would additionally like to thank my parents for their support and encouragement while I completed my degree, and for keeping a roof over my head. I would like to thank my sisters Amanda, Laura, and Nicole for being there for me when I needed anything. I would like to thank the Koumondurous family for everything they have done for me. Also, thank you to the rest of my family and friends. And finally, I would like to thank my soon to be husband, Philip Newmons, for his support, his love, and for so patiently waiting for me to finish my degree. I really appreciate everyone's help along the way, I could have never done this without each one of you, so again, I thank you!

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CHAPTER I

INTRODUCTION: THE PECAN NUT CASEBEARER, *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE)

The pecan nut casebearer, *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae) is an important, monophagous pest of pecan *Carya illinoinensis* (Wang) K. Koch (Fagales: Juglandaceae). Damage to pecans is a result of larvae burrowing into and feeding on nutlets. Two synthetic pheromones have been developed and are currently used to detect the flight of *A. nuxvorella* adults (Millar et al., 1996; Harris et al., 2008). The first pheromone developed (henceforth standard pheromone) consists of a single aldehyde (9*E*, 11Z)-hexadecadienal (9*E*, 11Z-16:Ald) (Millar et al., 1996). This pheromone has been used to attract male *A. nuxvorella* in the US for over 10 years, but interestingly, the standard pheromone fails to attract *A. nuxvorella* males in Mexico, even though *A. nuxvorella* is known to be present in Mexico. Due to the failure of the standard pheromone to attract *A. nuxvorella* in Mexico, the second *A. nuxvorella* pheromone (henceforth Mexican pheromone) has been developed (Harris et al., 2008). The Mexican pheromone (9*E*, 11Z)-hexadecadien-1-yl acetate (9*E*, 11Z-16:Ac): (9*E*, 11Z-16:Ald) consists of the same aldehyde component present in the standard pheromone plus an acetate component. This pheromone has been successful at catching moths in the US and in Mexico.

This thesis follows the style of the *Journal of Chemical Ecology*.

A. nuxvorella is multivoltine, typically having three to four generations per year, depending on geographic location (Neunzig, 1972). The first generation larvae are the most destructive to pecan crops due to the small size of the nutlets on which they feed. First generation larvae are capable of consuming 2-5 nutlets in a single cluster (Neunzig, 1972). Second and third generation larvae are less damaging and due to the increased size of the nutlet, they can complete development within 1-2 nutlets. *A. nuxvorella* females are capable of laying 150-200 eggs singly on individual nutlets (Bilsing, 1927). The newly hatched larvae move into the nutlets and begin to feed on the developing tissues of the pecan nut. *A. nuxvorella* larvae pupate within the concealment of a hollowed out nutlet.

The current integrated pest management (IPM) strategy for controlling *A. nuxvorella* consists of monitoring for this pest with pheromone baited sticky traps and using a degree day prediction model combined with a sequential sampling plan to ensure only needed insecticide treatments are applied at the correct time (Stevenson et al., 2003; Ring et al., 1983). These strategies have been helpful in timing pesticide applications, allowing for more efficient pesticide use. *A. nuxvorella* is most susceptible to pesticides as first instar larvae before entering the nutlet to feed. Thus, it is crucial that treatments be made before the larvae enter the nutlet and begin feeding. Pheromone traps are used to better predict the peak flight times of mating adults so that producers can use this information to spray for *A. nuxvorella* at optimal times as opposed to prophylactic control. The current IPM strategic plan for pecan has significantly reduced the amount of insecticides used to control *A. nuxvorella* compared to the amount of insecticides that

were applied when so called “calendar sprays” were used to control this pest. The reduced pesticide use increases the chances that foliar pecan pests, such as aphids, mites and leaf miners can be effectively controlled by natural enemies later in the pecan growing season (Harris et al., 1998).

The identification and synthesis of the *A. nuxvorella* pheromones is important to the IPM of this key pest of pecan. The discovery and production of *A. nuxvorella* pheromones has led to the implementation of better control practices due to more effective monitoring and the pheromones could possibly be used as a method of population reduction through pheromone based control. *A. nuxvorella* is a good candidate for this type of control because the adult males typically emerge from pupation a couple of days before the adult females. This would allow for a number of adult male *A. nuxvorella* to be captured in pheromone traps before the adult females emerge. Also, the current damage threshold for pecan nutlet damage is relatively high at 10% (Ring et al., 1989).

The presented thesis will offer the first genetic information for this important pest. This genetic information will be used to determine the existence of pheromone strains in *A. nuxvorella* (Chapter II). Likewise, genetic information will be used to determine the population structure of *A. nuxvorella* across the geographic distribution of this pest (Chapter III). The response behavior of *A. nuxvorella* when given a choice between the Mexican and standard pheromones will also be presented (Chapter IV). And finally, the relative abundance and a comparison of flight phenology of the two

pherotypes (i.e., conspecific males that show differential response to pheromone constructs) (Zada et al., 2008) at each of our study sites will be assessed (Chapter V).

CHAPTER II

SEARCHING FOR PHEROMONE STRAINS IN *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE)

Introduction

Two synthetic sex pheromones have been characterized and are currently used to detect the flight of the pecan nut casebearer *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae) (Millar et al., 1996; Harris et al., 2008). Sex pheromones are used by many insects to facilitate mate location and recognition (Löfstedt, 1993). In the majority of moth species, it is the female that produces the chemical signal and the male that responds. Sex pheromones are the primary source of communication among mating moths (Tamaki, 1985). The responder is capable of distinguishing among pheromones and is attracted to a very specific chemical signal produced by a conspecific female (Svensson, 1996).

There are several examples of lepidopterans that have polymorphic sex pheromones within the species. A thoroughly studied example is *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae): females of one pheromone race produce and males respond to a 3:97 E/Z-11-tetradecenyl acetate pheromone blend while in a second pheromone race, females produce and males respond to a 99:1 E/Z pheromone blend (Klun et al., 1973; Kochansky et al., 1975). Studies have found that *O. nubilalis* pheromone production and response can be attributed to insect genetics (Klun and Maini, 1979; Dopman et al., 2004) and to host plant use (Pelozuelo et al., 2004).

Likewise, pheromone races in *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) are linked to host plant preference (Groot et al., 2008). In contrast, an aberrant pheromone blend resulting from a single autosomal gene mutation was discovered in a laboratory population of *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) in which mutant females were unattractive to normal, wild type males (Haynes and Hunt, 1990). Two distinct pheromone blends and one intermediate pheromone blend were discovered for *Hemileuca eglanterina* Boisduval (Lepidoptera: Saturniidae) (McElfresh and Millar, 2001). It is believed that these two distinct pheromones are a result of reproductive character displacement and that the intermediate blend is a result of hybridization between the two pheromone races where they occur in sympatry (McElfresh and Millar, 2001). Other examples of moth species that exhibit polymorphic pheromone production include *Agrotis segetum* Denis & Schiffermüller (Lepidoptera: Noctuidae) (Löfstedt et al.,1986; Hannson et al., 1990), *Argyrotaenia velutinana* Walker (Lepidoptera: Tortricidae) (Miller and Roelofs, 1980), and *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) (Collins and Cardé, 1985). Each of these cases offers a unique angle to learn more about the evolution of chemical signaling in the Lepidoptera.

Löfstedt (1990) hypothesized that aberrant pheromone blends and polymorphic pheromone production in lepidopteran populations are maintained due to the existence of males that are able to respond to a wide range of pheromone blends. Although the specificity of sex pheromones can lead to reproductive isolation in insect species (Roelofs and Comeau, 1969), the evolution of sex pheromones is not well understood.

The objective of this study was to determine the existence of pheromone strains of *A. nuxvorella*. Amplified fragment length polymorphism (AFLP) markers were used to assess the existence of pheromone strains in this insect (Vos et al., 1995). AFLP markers have successfully been used to confirm the existence of biotypes or strains in other insect species (Cervera et al., 2000; Salvato et al., 2002; Zhu-Salzman et al., 2003; Busato et al., 2004; Zhang et al., 2005). The data presented in this study are the first genetic data available for this important pecan species. The hypothesis of this study is that *A. nuxvorella* pheromone strains (i.e., standard and Mexican) are reproductively isolated, thus genotypic differences are expected between the different pherotypes at each of our study sites.

Methods and Materials

AFLP markers (Vos et al., 1995) were used to detect differences in the genotypes of male *A. nuxvorella* that responded to different pheromones. AFLP markers were chosen because they are capable of surveying the whole genome of an organism when no prior genetic information is available. Additionally, they can be obtained fairly inexpensively and they produce many markers in a relatively short period of time (Pejic et al., 1998; Althoff et al., 2007).

Specimen Collection *A. nuxvorella* used for this study were collected from pheromone baited sticky traps (Trece Inc, Adair, OK) from 21 locations in the Southern US and Mexico (Figure 2.1). Each trap was baited with a rubber septum impregnated with 100

μg of synthetic pheromone. At each location three to six Trece Pherocon III™ sticky traps baited with synthetic standard pheromone lures and three to six sticky traps baited with synthetic Mexican pheromone lures were used to capture male moths. Traps were placed in pecan trees 2 meters above the ground and at least 50 meters from other traps. Trapped male moths were transferred to microcentrifuge tubes containing 70% alcohol at the collection locations. Specimens were then sent to Texas A&M University in College Station, Texas, and stored at -80 ˚C until used for genetic analysis. Samples were collected for two consecutive years (between the months of April and September of 2007 and 2008). A total of 181 specimens were selected at random for AFLP analysis.

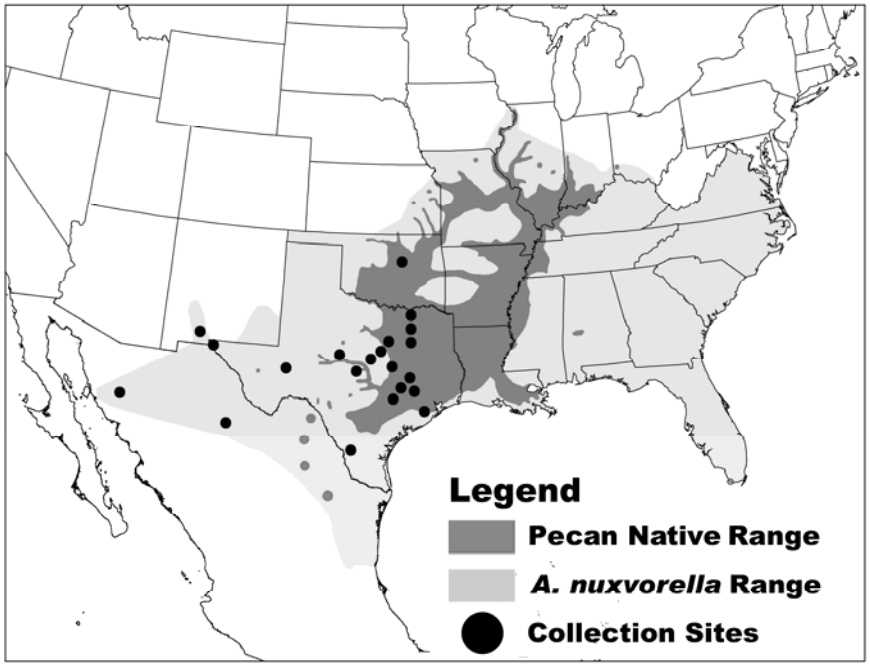

Fig. 2.1 Native pecan distribution, range of *A. nuxvorella,* and collection sites used to determine if pheromone strains exist. Pecan is native to the alluvial plains of the south-central US and mountain valleys of Mexico, but has expanded due to cultivation. *A. nuxvorella* is present in pecan growing regions in the US east of the Rocky Mountains and in pecan growing regions in northern Mexico

DNA Extraction Microcentrifuge tubes containing *A. nuxvorella* male individuals selected for molecular analyses were taken out of -80 °C storage and placed in liquid nitrogen. Entire individuals were frozen in liquid nitrogen and crushed to start DNA extraction procedures. Only a sub-sample of specimens collected per each pheromone lure at each of the study sites were used for molecular marker studies and the remaining specimens were kept as vouchers. DNA was extracted using a QIAGEN DNEasy Tissue Kit (Qiagen Inc., Valencia, CA) following Qiagen recommended protocol (Qiagen, 2002). The quantity (ng of nucleic acid per µl of solution) and quality (ratio of sample absorbance at 260 and 280 nm) of DNA was measured using a NanoDrop® spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE)

AFLP Development AFLP markers were used to determine genetic differentiation among *A. nuxvorella* from the presumed pheromone strains at each of the 21 collection locations. Digestion of genomic DNA by the restriction enzymes *Eco*RI and *Mse*I and ligation of oligonucleotide adaptors compatible with these endonucleases (Applied Biosystems, Foster City, CA) were accomplished in a single reaction mixture. Each reaction aliquot contained approximately 1100 ng of template DNA. Pre-selective polymerase chain reaction (PCR) amplification was performed using the Applied Biosystems AFLP Preselective Mix (Applied Biosystems, Foster City, CA). The PCR program for pre-selective amplification was: 95 °C for 1 min followed by 20 cycles of 95 °C for 10 sec, 56 °C for 30 sec, and 72 °C for 1 min 30 sec with a final hold at 75 °C for 5 min. All samples were stored at 4 °C following pre-selective amplification on a

thermal cycler and before selective amplification. The amplified product was then diluted 20-fold using 15 nM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. For the selective amplification of restriction fragments, pre-prepared custom primers for recognition of EcoRI and MseI adaptors were used. The four primer pairs selected were M-CAT and E-ACT, M-CTC and E-AAC, M-CAC and E-ACG, and M-CAA and E-ACT. Fragments were visualized by attaching a fluorescent dye to the 5' end of each EcoRI selective amplification primer with no modification made to the MseI primer. The PCR program for the selective amplification process consisted of an initial warm-up at 95 °C for 30 sec, 12 cycles of 95 °C for 10 sec, 65 °C for 40 sec with a lowering of 0.7 °C per cycle, 72 °C for 1 min 30 sec, followed by 35 cycles of 95 °C for 11 sec, 56 °C for 40 sec, 72 °C for one min 30 sec and finally a hold of 75 °C for 5 min before storing the samples at 4 °C.

Data Analyses AFLP markers were separated by capillary electrophoresis by a 3100 Genetic Analyzer from Applied Biosystems (Foster City, CA) and analyzed using GeneMapper® software (Applied Biosystems, 2005). Markers with a dye signal larger than 100 luminescent units were considered as present. Each AFLP marker was considered a locus and was assumed to have two possible alleles (represented in binary form, i.e., 0 for no allele present and 1 for a present allele) The number of specimens and primer combinations necessary to detect genetic differences in this study was assessed using SESim values (Medina et al. 2006). The four primer pairs mentioned above were considered sufficient based on a SESim < 0.05.

The possibility of two pheromone strains of *A. nuxvorella* was investigated using STRUCTURE 2.2 (Pritchard et al., 2000; Falush et al., 2007). STRUCTURE 2.2 is a Bayesian inference method that uses a Markov chain Monte Carlo (MCMC) algorithm to cluster individuals into populations and measures the probability that an individual is a member of different populations based on genotypic data (Pritchard et al., 2000; Falush et al., 2007). The STRUCTURE 2.2 burn-in period was fixed at 10,000 with a run length of 10,000 under the admixture model with correlated and uncorrelated allele frequencies. To determine the number of populations present within the data (K), twenty replications were completed for each K value between 1 and 5. The number of populations (K) indicated by STRUCTURE 2.2 was verified using the ΔK method of Evanno et al. (2005).

Results

A total of 181 individuals from 21 locations throughout the distribution of *A. nuxvorella* were used for AFLP analyses. The average concentration of DNA for the 181 specimens was 110.69 ng of nucleic acid per μl of solution. The average quality of DNA samples as measured by the ratio of light absorbance at 260 and 280 nm was 2.00. The four primer combinations used yielded 483 polymorphic AFLP markers. This number of individuals and AFLP markers resulted in a SESim value of 0.0262. A SESim value of <0.05 indicates that the sample size of individuals and genetic markers is sufficient to accurately capture the genetic variability of the two postulated strains of *A. nuxvorella* at the geographic scale of this study (Medina et al., 2006). When the Evanno ΔK method (Evanno et al., 2005) was used to predict the number of populations generated by STRUCTURE 2.2, the results indicated that there were 3 distinct populations under the

admixture and correlated allele frequencies model (Figure 2.2).The first (US 1) and second (US 2) clusters contained all of the individuals collected in pheromone traps in the US. The first cluster (US 1) contains individuals collected from each of the US collection sites with pheromone traps baited with the Mexican pheromone and pheromone traps baited with the standard pheromone. Similarly, the second cluster (US 2) contains individuals from each of the US collection sites and individuals from pheromone traps baited with the Mexican and standard pheromone. The third cluster (MEX) is made up of all of the individuals collected from pheromone traps in Mexico. Due to the inability of the standard pheromone to attract *A. nuxvorella* males in Mexico, all of the individuals in the third cluster (i.e., MEX) were captured using the Mexican pheromone only. Thirteen markers were only present in individuals belonging to the Mexican cluster and absent in all individuals in the two US clusters. One of the markers that was absent in all US collected individuals was present in all but one of the Mexican collected individuals, and could potentially be used as a diagnostic band for the Mexican population.

Discussion

The results of this study indicate that there is no evidence supporting pheromone strain formation among populations of *A. nuxvorella*. There was, however, a distinct difference between *A. nuxvorella* captured in Mexico and the US. The westward expansion of the pecan industry in the US and Mexico has recently been followed by the expansion of *A. nuxvorella* into areas where pecan is not indigenous, but has been

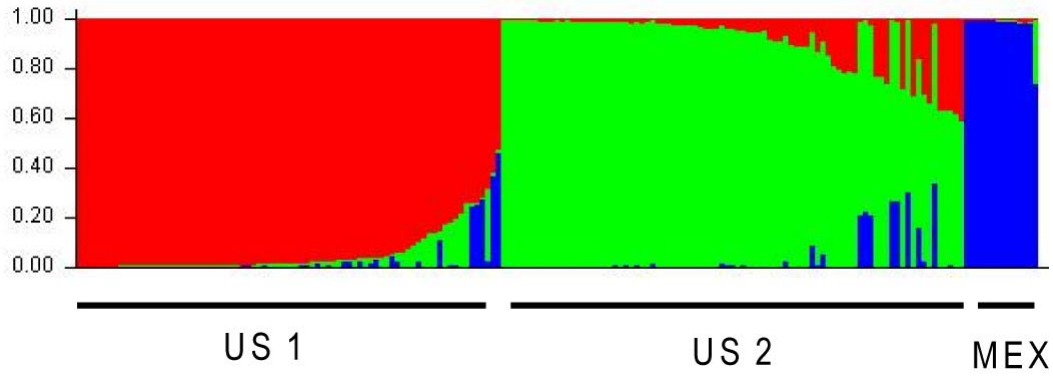

Fig 2.2 Results of STRUCTURE 2.2 (Pritchard et al., 2000) when used to determine if pheromone strains exist in *A. nuxvorella*. Each vertical line represents one individual and the proportion of red (US 1), green (US 2), and blue (MEX) corresponds to the probability that an individual is a member of a particular population. The most likely number of clusters was 3 according to the ΔK method of Evanno et al. (2005)

cultivated since the late 1800's (Brison, 1974). This pest was found infesting pecan plantings in the El Paso Valley of Far West Texas for the first time in 1988 (Harris et al., 1988). *A. nuxvorella* is autochthonous in some regions of Mexico and expanded to pecan plantings in the Mexican state of Sonora as recently as 2001 (Fu Castillo et al., 2005). Most likely this westward expansion of *A. nuxvorella* into Sonora, Mexico is a result of the importation of infested plant materials from Chihuahua into these areas. Genetic differences between the US and Mexican moths are most likely due to the geographic isolation of pecan in Mexico from those in the US.

It was initially hypothesized by Harris et al. (2008) that the Mexican pheromone originated in Mexico as a mutant form of the standard pheromone and was introduced and established in the US via increased commerce between the two nations. An alternative hypothesis is that the Mexican pheromone is the ancestral form. North

America has experienced numerous glacial periods since the late Pliocene, with the most recent glacial period occurring in the Pleistocene. The Juglandaceae, the family to which pecan belongs, had a northern limit of 34˚ 16,000 years ago, but as recently as 8,000 years ago has expanded northward to its current northern limit of 45˚ (Delcourt and Delcourt, 1987). The standard pheromone may have evolved from a mutation of the Mexican pheromone and become established in isolated population of *A. nuxvorella* during a period of glacial retreat when pecan stands occurred in isolated pockets. As pecan populations began to expand after the most recent glacial retreat the two pherotypes may have introgressed into their current sympatric state in the US. As of yet, the two pherotypes do not occur sympatrically in Mexico.

Additionally, the absence of the standard *A. nuxvorella* pheromone in Mexico may be attributed to the fact that the sugarcane borer, *Diatraea saccharalis* F. (Lepidoptera: Crambidae) also produces the same sex pheromone (i.e., (9*E*, 11Z)-hexadecadienal (9*E*, 11Z-16:Ald)) (Santangelo et al., 2001). It is possible that *A. nuxvorella* in Mexico are unresponsive to this pheromone as a mechanism to avoid cross attraction between the two species. If male *A. nuxvorella* were attracted to female *D. saccharalis,* the result would be wasted reproductive effort for those individuals attracted to incompatible females.

D. saccharalis is one of the most damaging stalk boring pests of corn in Mexico (Maredia and Mihm, 1991). Corn is grown in every state in Mexico, thus there is considerable overlap between pecan growing regions and corn growing regions in Mexico leading to *A. nuxvorella* and *D. saccharalis* occurring in sympatry. Further, in

Mexico, there is a great deal of temporal overlap between *A. nuxvorella* and *D. saccharalis*. The adults of the first generation of *A. nuxvorella* begin emerging in late April and the flight of the final generation ends in early September (Fu Castillo et al., 2005). In northern Mexico, *D. saccharalis* adults begin emerging in March and subsequent generations continue their flights into September (Rodriguez del Bosque et al., 1995).

D. saccharalis is also a major pest of corn, rice, and sugarcane in the US, but it is only a pest in the warmer parts of the Gulf Coast States (i.e. Florida, Louisiana, and Texas) (Capinera, 2007). *D. saccharalis* adults begin emerging in April or May in the Gulf Coast States and subsequent generations continue flight into the autumn months (Capinera, 2007). In the US, *A. nuxvorella* adults begin flying in late April and subsequent generations continue into October (Bilsing, 1926; Stevenson et al., 2003; Harris, unpublished data). Thus, although there is also a temporal overlap between *D. saccharalis* and *A. nuxvorella* in the US, *A. nuxvorella* in the US are somehow able to maintain the integrity of both chemical signals, probably because the spatial overlap of these two pest species is limited to the Gulf Coast Region.

The two sympatric US populations of *A. nuxvorella* (see Figure 2.2) are both comprised of individuals captured at each location with each of the pheromones. This is an indication that male *A. nuxvorella* in the US are able to recognize and respond to both of the pheromones produced by *A. nuxvorella*. This widened response by moths in the US can be explained by Phelan's asymmetric tracking hypothesis which states that male moths will be most sensitive to the most common pheromone blend present within a

population, but that male response should be wide enough to recognize potential mates of the same species, even if the calling female belongs to a different pheromone race (Phelan, 1992). If this is the case for *A. nuxvorella*, it is likely that there is a pheromone polymorphism in females, but there is not a response polymorphism in males.

Liu and Haynes (1994) showed that *T. ni* males from what they refer to as mutant sex pheromone producing colonies preferred females that produced normal, wild type, sex pheromones, but after 49 generations the males from mutant colonies displayed a widening response that included both pheromone blends and began responding to normal and mutant pheromones with equal frequency. Another example of a wide response to sex pheromones is exhibited between *O. nubilalis* and its congener *O. furnacalis* Guenée (Lepidoptera: Crambidae). These two species are closely related and showed cross attraction between species when tested in a wind tunnel (Roelofs et al. 2002; Linn et al., 2003; Linn et al., 2006).

It is possible to have two pherotypes within the same species that are not reproductively isolated, and there are examples of this in the Lepidoptera (McElfresh and Millar, 2001; Liu and Haynes, 1994). The two pherotypes of *A. nuxvorella* could be two different phenotypes (much the same as color or size) that although explained by differences in one or few genes, do not lead to the reproductive isolation of the two *A. nuxvorella* pherotypes or the formation of distinct pheromone strains. Thus, it is possible that in Mexico, *A. nuxvorella* respond to the Mexican pheromone because genes associated with recognition of the Mexican pheromone were selected for while genes responsible for the response to the standard pheromone were selected against, possibly

because of signal competition with *D. saccharalis*. In the US, moths are attracted to both the standard pheromone and the Mexican pheromone perhaps because they experience less selective interference from *D. saccharalis.*

Not much is currently known about the mating behavior of *A. nuxvorella*, and the question still remains as to why male *A. nuxvorella* in the US are responsive to the Mexican and standard pheromones while in Mexico the standard pheromone seems to be absent. It is possible that the Mexican pheromone evolved long ago to reduce interspecific competition between *A. nuxvorella* and *D. saccharalis*. Further research is needed in order to explain the existence of the two characterized *A. nuxvorella* pherotypes.

CHAPTER III

POPULATION STRUCTURE OF *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE) THROUGHOUT ITS GEOGRAPHIC DISTRIBUTION

Introduction

Acrobasis nuxvorella Neunzig (Lepidoptera: Pyralidae) is monophagous on pecan. Thus, the geographic distribution of *A. nuxvorella* is linked to the distribution of its host-plant species, *Carya illinoinensis*. Pecan is indigenous to the alluvial plains in the south-central US and mountain valleys of Mexico (Harris, 1983). *A. nuxvorella* is distributed throughout the pecan growing regions east of the Rocky Mountains in the US and throughout northern Mexico. The aboriginal range of *A. nuxvorella* reaches from Louisiana west to the eastern edge of New Mexico and north to Illinois in the US and as far west as Chihuahua and south to Oaxaca in Mexico (Harris, 1983). The expansion of the pecan industry in the US and Mexico has recently been followed by the expansion of *A. nuxvorella* into areas where pecan is not indigenous (Harris et al., 2008), but has been cultivated since the late 1800's (Brison, 1974). Recently, *A. nuxvorella* has expanded to pecan growing regions in Sonora, Mexico (Fu Castillo et al., 2005) and the El Paso Valley in far west Texas (Harris et al., 1988) (Figure 3.1).

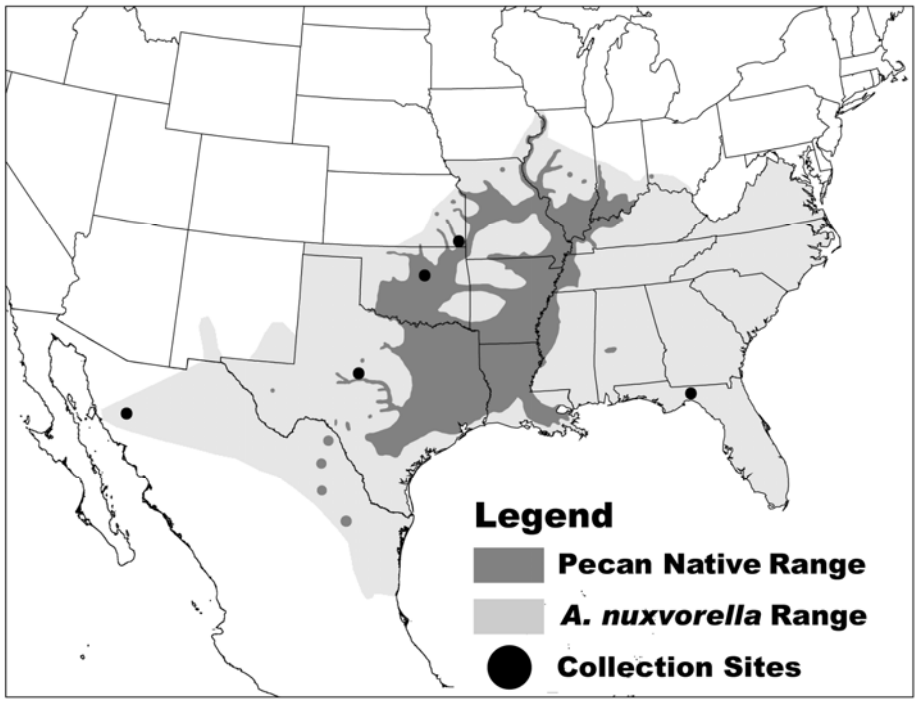

Fig. 3.1 Native pecan distribution, range of *A. nuxvorella,* and collection sites used to assess population structure. Pecan is native to the alluvial plains of the south-central US and mountain valleys of Mexico, but has expanded due to cultivation. *A. nuxvorella* is present in pecan growing regions in the US east of the Rocky Mountains and in pecan growing regions in northern Mexico

The geographic distribution of a species may contain a single panmictic population or instead can be comprised of several populations. If several populations can be found within a species' geographic distribution, these populations are described as geographically structured. Geographic population structure is reflected in the distribution of genetic variation within and among populations and can be indirectly inferred through a number of molecular methods (Roderick, 1996). Additionally, the degree of genetic differentiation within and among populations can be inferred using molecular markers

(Hartl and Clark, 1997). The determination of the population structure of pest species has many practical applications including verification of pheromone races, biotypes and host races within a species (Cervera et al., 2000; Salvato et al., 2002; Zhu-Salzman et al., 2003; Busato et al., 2004; Zhang et al., 2005; Drés and Mallet, 2002), and identification of the origin of introduced pests (Bogdanowicz et al., 1997; Liu et al. 2009).

Understanding the population structure of insect pests is necessary in order to apply the most efficient monitoring tools and pest management strategies to aid in decision making because pest populations bearing a high degree of genetic variation may respond differently to control regimes (Menken and Raijman, 1996). For example, determining the population structure for insect pests is important because it might provide insight into the patterns of localized insecticide resistance (Labbe, 2005). The degree of dispersal exhibited within a pest species can also be inferred from population structure studies. For instance, Caprio (2001) showed that the degree of gene flow between insect subpopulations could greatly affect the time it took insecticide resistance to develop. Similarly, the boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), was found to have great dispersal capabilities that may have a negative impact on the eradication efforts for boll weevil because it is possible for *A. grandis* to migrate from areas in which eradication efforts are not enforced, such as Mexico, into the eradication zones within the US (Kim and Sappington, 2006).

The objective of this study was to assess the population structure of *A. nuxvorella* across its geographic range using amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995). AFLP markers have been used successfully to determine the

population structure of other Lepidoptera including *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae) (Mozaffarian et al., 2008), *Lymantria dispar* L. (Lepidoptera: Lymantriidae) (Reineke, 1998) and *Thaumetopoea pityocampa* Denis & Schiffermüller (Lepidoptera: Notodontidae) (Salvato et al., 2002).

The population structure of *A. nuxvorella* is currently unknown. The presence or absence of population structure within *A. nuxvorella* collected from different study sites will indicate the degree of reproductive isolation among them. If reproductive isolation is present, it might be due to the existence of some sort of geographic (Gerlach and Musolf, 2000; Stamford and Taylor, 2005; Narum et al., 2006) or ecological barrier (Storfer, 1999), limited dispersal abilities (Bacilieri et al., 1994; Arnaud et al., 2001; Salgueiro et al., 2003; Sundstrom et al., 2003), adaptation to local conditions (Grahame et al., 2006), or due to a founder effect resulting from introduction into a new area (Sakai et al., 2001). Lack of population structure can be interpreted as wide dispersal capabilities and consequently lack of reproductive isolation between *A. nuxvorella* populations (Delmotte et al., 2002).

Methods and Materials

Population structure of *A. nuxvorella* was assessed by analyzing AFLP markers using STRUCTURE 2.2 (Pritchard et al., 2000, Falush et al., 2007). *A. nuxvorella* used for this study were collected from pheromone baited sticky traps (Trece Inc., Adair, OK) from 5 representative locations within the geographic distribution of *A. nuxvorella* (i.e., Jefferson Co., Florida; Payne Co., Oklahoma; Hermosillo, Sonora, Mexico; Comanche

Co., Texas; Labette Co., Kansas) (Figure 3.1). Each trap was baited with a rubber septum impregnated with 100 μg of synthetic pheromone. At each location three to six Trece Pherocon III™ sticky traps baited with synthetic standard pheromone lures and three to six sticky traps baited with synthetic Mexican pheromone lures were used to capture male moths. Traps were placed in pecan trees 2 meters above the ground and at least 50 meters from other traps. Trapped male moths were transferred to microcentrifuge tubes with 70% alcohol at the collection locations. Specimens were then sent to Texas A&M University in College Station, Texas and stored at -80 ˚C until ready for genetic analysis. Samples were collected for two consecutive years between the months of April and September of 2007 and 2008, with the exclusion of the collection site in Labette Co., Kansas, where *A. nuxvorella* were only collected during 2009. A total of 30 specimens per location per year were selected at random for AFLP analysis. Pheromone lure type used to capture moths was not taken into account when randomly selecting individuals because previous studies found no genetic differences between pherotypes (See Chapter II).

DNA was extracted and AFLP markers were obtained following the protocols described in Chapter II Methods section (page 7) AFLP data were analyzed using STRUCTURE 2.2 (as described in Chapter 2) and F_{ST} values (Hartl and Clark, 1997; Wright, 1921) were calculated among individuals from different locations using AFLP-SURV 1.0 (Vekemans, 2002). The parameters used in AFLP-SURV 1.0 included the Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999) and Hardy-Weinberg genotypic proportions were assumed.

Results

A total of 244 individuals from 5 representative locations within the *A. nuxvorella* geographic distribution range (Figure 3.1) were used for AFLP analyses. The average concentration of DNA for the 244 specimens was 148.83 ng of nucleic acid per µl of solution. The average quality of DNA samples as measured by the ratio of light absorbance at 260 and 280 nm was 2.024. The four primer combinations used yielded 567 polymorphic AFLP markers. This number of individuals and AFLP markers resulted in a SESim value of 0.022. A SESim value of <0.05 indicates that the sample size of individuals and genetic markers is sufficient to accurately capture the genetic variability of the *A. nuxvorella* populations studied (Medina et al., 2006).

The Evanno ΔK method (Evanno et al., 2005) was used to predict the number of populations generated by STRUCTURE based on the genetic variation found in the data. This method indicated that there were 5 distinct populations under the admixture and correlated allele frequencies model (Figure 3.2). The relative abundance of individuals belonging to the 5 distinct identified populations varied across the geographic distribution of *A. nuxvorella* (Figure 3.3). The first population was mostly distributed in the central US. It consisted of 22 male *A. nuxvorella* from Payne Co., Oklahoma, 1 individual from Jefferson Co., Florida, 2 individuals from Hermosillo (Sonora, Mexico), and 2 individuals from Comanche Co., Texas. Population 2 was exclusively distributed in the Eastern US. It consisted of 41 individuals from Jefferson Co., Florida. Population 3 was exclusively distributed in Western Mexico. It consisted of 46 individuals from

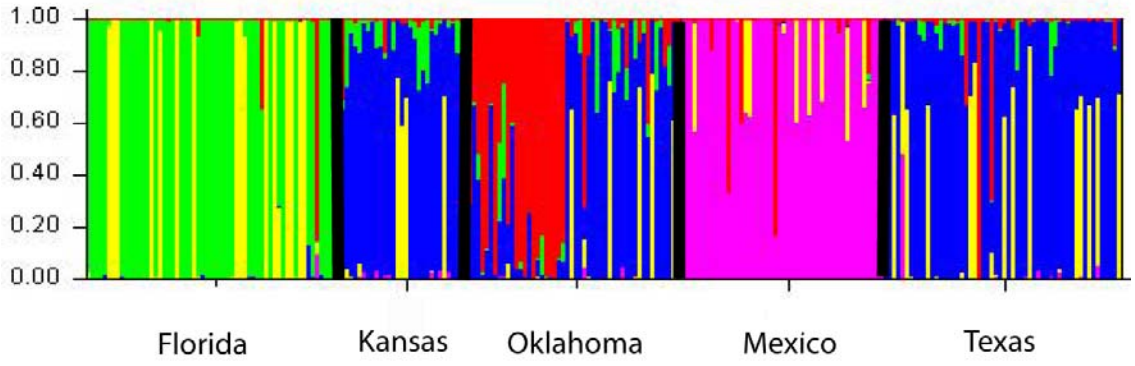

Fig. 3.2 Results of STRUCTURE 2.2 (Pritchard et al., 2000) when used to assess population structure of *A. nuxvorella*. Each vertical line represents one individual and the proportion of green (population 1), blue (population 2), yellow (population 3), pink (population 4), and red (population 5) corresponds to the probability that an individual is a member of a particular population. The most likely number of clusters was 5 according to the ΔK method of Evanno et al. (2005)

Hermosillo, Sonora, Mexico. Population 4 was distributed in the central and eastern US. It consisted of 14 individuals from Comanche Co. Texas, 16 individuals from Jefferson Co., Florida, 5 individuals from Payne Co. Oklahoma, and 4 individuals from Labette Co., Kansas. Population 5 was exclusively present in the central US. It consisted of 40 individuals from Comanche Co., Texas, 24 individuals from Payne Co., Oklahoma, and 26 individuals from Labette Co., Kansas.

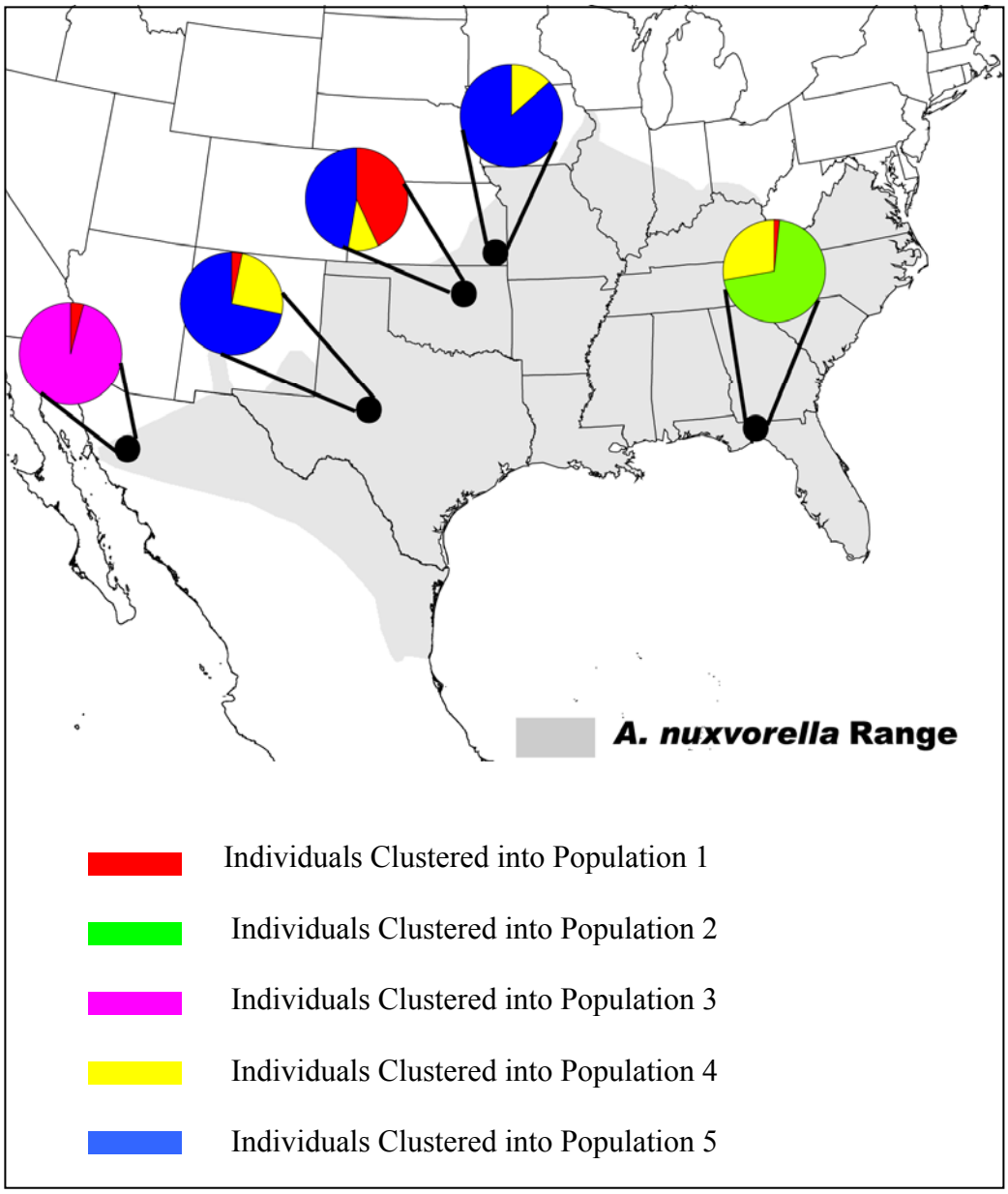

Fig. 3.3 Map showing the geographic distribution of *A. nuxvorella* in North America with pie-diagrams indicating the proportion of individuals belonging to different populations at each collection site as determined by STRUCTURE 2.2 (Pritchard et al 2000)

F_{ST} values were calculated in order to measure the amount of genetic divergence among the 5 collection sites. The F_{ST} value among populations was 0.1717 ($p < 0.05$). The pairwise F_{ST} values between the 5 collection sites are presented in table 3.1.

Table 3.1 Pairwise F_{ST} values between the 5 collection sites

	FL	KS	OK	MX	TX
FL	-				
KS	0.0917				
OK	0.1023	0.0425			
MX	0.3578	0.2949	0.2920		
TX	0.1096	0.0236	0.0455	0.2863	-

FL- Jefferson Co., Florida
KS- Labette Co., Kansas
OK- Payne Co., Oklahoma
MX- Hermosillo, Sonora, Mexico
TX- Comanche Co., Texas

Discussion

The results of AFLP analyses indicate a high degree of genetic structure in *A. nuxvorella* across its geographic distribution ($F_{ST} = 0.1717$). The male *A. nuxvorella* collected in Hermosillo (Sonora, Mexico), are genetically differentiated from the *A. nuxvorella* from the other four collection sites (Table 3.1). These data are corroborated by the results of cluster analysis that place all but 2 of the Mexican individuals into the Mexican population (i.e., population 3). This Mexican population represents the western most distribution of *A. nuxvorella* and also represents the most recent known *A. nuxvorella* infestation. *A nuxvorella* was found infesting pecan in Sonora, Mexico for the first time

in 2001, presumably as a result of infested plant materials from Chihuahua, Mexico (Fu Castillo et al., 2005). It is likely that the high degree of genetic homogeneity observed in this population is due to a genetic bottleneck that occurred at the time of introduction. Introduction of a species into new areas are prone to founder effects because the invasions usually only involve a small number of individuals (Sakai et al., 2001). Genetic differences in Mexican collected *A. nuxvorella* can also be attributed to the fact that this population is found on pecan plantings that are non-native and are geographically isolated from the remainder of the areas sampled for this study. This geographic isolation is due to non-contiguous pecan plantings outside of the native pecan range. This isolation may restrict gene flow between the Mexican population and the populations found within the native pecan range. Within its native range, pecan is contiguous along river valleys and much of the pecan production within this range utilizes native pecan trees by thinning out competing vegetation (Harris, 1983). Thus, in the native range of pecan, the habitat utilized by *A. nuxvorella* is less fragmented. In Mexico, geographic isolation is increased by the Sierra Madre Occidental Mountain Range. This mountain range has been shown to pose a geographic barrier to gene flow in other species (Swenson and Howard, 2005). Furthermore, the large distance between this sampling location in Mexico and the three locations in the central US is another factor that serves to restrict gene flow between these populations.

A. nuxvorella collected from Jefferson Co., Florida were moderately differentiated from the other 3 US populations. The *A. nuxvorella* population in Florida is not within the native range of this pest, and like in the Mexican population, the

homogeneity in genetic composition of the individuals in Florida is probably due to a founder effect that occurred when *A. nuxvorella* first infested this area. This population is also restricted from exchanging genes with the central US populations by geographic isolation due to considerable habitat fragmentation in areas where pecan is not native, as well as by the relatively large distance between Florida and the other 3 locations within the US.

The results indicate that there is very little genetic divergence between the three central US populations (Comanche Co., Texas, Payne Co., Oklahoma and Labette Co., Kansas) (Table 3.1). This is expected because all three populations occur within the native pecan range (Figure 3.1), presumably the center of origin for *A. nuxvorella*. This idea is supported by the high level of genetic diversity of *A. nuxvorella* within the native pecan range (Kim et al., 2006; Althoff and Pellmyr, 2002). The lack of genetic isolation within these populations is indicative of frequent gene flow between *A. nuxvorella* inhabiting the central US. As mentioned above, this can be attributed to the decreased habitat fragmentation in areas where pecan is native as opposed to increased habitat fragmentation in areas where pecan has been introduced for cultivation.

The results of this study indicate that *A. nuxvorella* populations in areas where pecan is not native but has been cultivated are genetically isolated from the populations that occur within the native distribution of pecan. There is also some indication that long range dispersal of this insect is limited to areas where pecan is present contiguously. The genetic data presented in this chapter have practical applications for the management of *A. nuxvorella*, these data can be used in the future to determine the origin of future

infestations of *A. nuxvorella* at new infestation locations, it can suggest if control measures will be effective on the entire population (if panmictic) or if it will be useful to consider differential response of distinct populations to control practices (e.g., differential resistance to insecticides and/or to natural enemies at different locations). Future studies that include more *A. nuxvorella* collection sites within the Central and Eastern US will be beneficial in determining if there is further sub-structuring. Data from more geographic locations within the Eastern and Central US will more conclusively assess the dispersal capabilities of *A. nuxvorella.* Furthermore, data from more geographic locations will offer insight into whether or not the Appalachian Mountain Range serves as a geographic barrier much the same as the Sierra Madre Occidental Mountain Range. This knowledge would allow us to determine if the Rocky Mountain Range will serve as an effective geographic barrier and prevent the westward expansion of *A. nuxvorella* into pecan growing regions west of the Rocky Mountains where this species is currently not established.

CHAPTER IV

EVIDENCE OF ASYMMETRIC TRACKING OF FEMALE PRODUCED SEX PHEROMONES BY *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE) MALES

Introduction

Sex pheromones are the primary source of communication among mating moths (Tamaki, 1985; Svensson, 1996). Two pheromones that are produced by *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae) females in order to attract potential male mates have been characterized (Harris et al., 2008; Millar et al., 1996). The standard pheromone was identified to be (9*E*, 11*Z*)-hexadecadienal (9*E*, 11*Z*-16:Ald) (Millar et al., 1996) and the Mexican pheromone was identified as (9*E*, 11*Z*)-hexadecadien-1-yl acetate (9*E*, 11*Z*-16:Ac): (9*E*,11*Z*-16:Ald) (Harris et al., 2008). In Mexico moths are attracted to only the Mexican pheromone, while in the US male moths are attracted to both the standard and Mexican pheromones (Millar et al., 1996; Harris et al., 2008). Because no genetic differences were found to suggest pheromone strain formation in *A. nuxvorella* in the US (Chapter II), it is hypothesized that the males of this species in the US are able to recognize and respond to both of the pheromones produced by *A. nuxvorella* females. While, in contrast, it is hypothesized that moths from Mexico will only respond to the Mexican pheromone, because the standard pheromone has never been successful at capturing moths in Mexico.

As discussed in chapter II, there are well-studied examples of lepidopterans that exhibit polymorphic sex pheromone production within a species (Klun et al., 1973; Kochansky et al., 1975; Groot et al., 2008; Haynes and Hunt, 1990; McElfresh and Millar, 2001; Löfstedt, 1986; Hannson et al., 1990; Miller and Roelofs, 1980; Collins and Cardé, 1985). A widely accepted hypothesis regarding moth sex pheromones is that populations experience stabilizing selection where male moths are tuned in to one particular pheromone blend (Baker, 2002). One major flaw of this hypothesis is that it does not explain how new sex pheromone blends may come to exist within the same species (Löfstedt, 1993; Phelan, 1992). If males of a species are hardwired to respond only to one very specific sex pheromone blend, those females producing aberrant blends would be unable to attract a conspecific mate. If females with aberrant pheromone blends are unable to produce offspring, the new pheromone blend will be unlikely to persist in the population.

The asymmetric tracking hypothesis (Phelan, 1992) offers a solution to explain the existence of different pherotypes within the same species. The asymmetric tracking hypothesis states that (i) female moths will assume the least costly role in mate signaling and finding, (ii) female moths will experience very weak stabilizing selection to produce the most common pheromone present in a population and thus, there is expected to be variation from the norm, and (iii) male moths will be most sensitive to the most common pheromone blend present within a population, but male response should be wide enough to recognize potential mates of the same species, even if the calling female belongs to a different pheromone race. These predictions contend that sexual selection is inversely

correlated to the degree of sexual asymmetry (i.e. when one sex invests significantly more energy or resources into reproduction) in parental effort in moths (Phelan, 1992).

The objective of this study was to determine if male *A. nuxvorella* individuals show differential response to the two pheromones available, or if individual males are capable of recognizing and responding to both of the pheromones. What we currently know about the response of *A. nuxvorella* to the two pheromones is based on pheromone trapping which only tells us that the male is responsive to the pheromone with which it was trapped. It is known that *A. nuxvorella* will mate multiple times (Calcote et al., 1984). Testing the attraction of individual males to the two available pheromones multiple times will allow us to determine how *A. nuxvorella* will behave when given multiple opportunities to choose a pheromone, as is the case in their natural setting. The results of this study will indicate whether or not pheromone mediated mate recognition in *A. nuxvorella* is influenced by asymmetric tracking by males.

Methods and Materials

Insects The *A. nuxvorella* used for testing were collected as larvae from infested nutlets in College Station, Texas, USA and in Hermosillo, Sonora, Mexico. Larvae were taken from pecan orchards back to the laboratory where they were placed individually into 16 ounce plastic rearing containers (Newspring DELItainer®, Pactiv Corp., Lake Forest, IL). Due to the fact that *A. nuxvorella* larvae will not feed on an artificial diet, fresh, undamaged nutlet clusters were provided to larvae in order to complete development. Uninfested nutlet clusters for larval feeding were cut from pecan trees and the damaged

petiolules were sealed with paraffin wax to prevent fungal growth and desiccation. Larvae were kept in a rearing room maintained at an average temperature of 30 ˚C, with a daytime high of 34 ˚C and a nightly low of 26 ˚C. The average relative humidity was 33.5%.

Infested nutlets were checked twice weekly for pupae. Once in the pupal stage, *A. nuxvorella* were moved to another rearing room where they were conditioned to a 14:10 L/D photoperiod opposite of that which occurs naturally so that moths could be tested during the day. This room was maintained at an average temperature of 24 ˚C, with a daytime high of 26 ˚C and a nightly low of 22 ˚C. The average relative humidity was 34.5%. This is the same room in which males were subjected to olfactometric testing. Once adults emerged, they were temporarily incapacitated with CO_2 and sexed using sex-specific characters of the genitalia (Fu Castillo, pers. comm.). Only males were retained for olfactometric testing. Nourishment was provided to *A. nuxvorella* males in the form of a honey water (1:1 ratio of honey and distilled water) soaked piece of filter paper placed in a plastic weighing boat.

Pheromone Lures Synthetic pheromone lures of the Mexican pheromone and standard pheromone were used for olfactometric testing. Pheromone lures were prepared by J.G. Millar's laboratory at the University of California Riverside. The pheromone lures consisted of 100 µg of synthetic *A. nuxvorella* pheromone impregnated into rubber septa.

Olfactometric Testing Moths were tested in a y-tube olfactometer (Analytical Research Systems, Inc., Gainesville, FL) the first scotophase after eclosing. Each moth was tested once a day for five consecutive days or until death occurred, whichever came first. Moths were tested starting at 3 hr after the onset of scotophase when they become most active (Calcote et al., 1972). The testing room had no windows to simulate the lack of sunlight at night, and a red light was installed in the room for illumination. The olfactometer delivered air from the opening of each arm past the bifurcation of the y-tube towards the opening at the opposite end at a pressure of 18 psi.

A. nuxvorella males were tested individually. Each male was placed in the olfactometer along with one standard pheromone lure in one arm of the y-tube and one Mexican pheromone lure in the other arm of the y-tube. Pheromones were placed randomly into an arm each day by rotating the y-tube 180˚. Males were taken from their containers and placed into the y-tube for testing. Each male was given 15 min to choose a pheromone and a positive response was counted after spending at least two min past a designated point one inch past the point of bifurcation.

Statistical Analyses Total counts were converted into percentages for graphical display. First choice by *A. nuxvorella* males were analyzed using the χ^2 test for equal proportions using SPSS 15.0 statistical analysis software (SPSS, 2006).

Consistency of response was tested using χ^2 with SPSS 15.0 statistical analysis software (SPSS, 2006) for US collected moths. Due to a small sample size for the Mexican collected moths, consistency of response was analyzed using the Quantitative

Skills Exact Multinomial Test 2.2 software (Quantitative Skills, 2008). Expected values for each category of response behavior (e.g., choosing the Mexican pheromone only, choosing the standard pheromone only, or choosing both pheromones during different testing periods) were calculated as follows:

$$E = \sum \frac{\left(n_i\left(k_i\right) + n_{i-1}\left(k_{i-1}\right) + n_{i-2}\left(k_{i-2}\right)...n_2\left(k_2\right)\right)}{t}$$

Where E is the expected proportion of moths in a response behavior category, n is the number of individuals within a particular response category, t is the total number of individuals, and k is the summation of all probabilities of a specific sequence of events (i.e. responses) that lead to a specific response behavior category and was calculated as follows:

$$k = \sum \frac{1}{p_c}$$

Where p is the probability of a specific sequence of responses by an individual, and c is the response category. The probability of a specific sequence of responses by an individual was calculated as follows:

$$p = \frac{1}{s^i}$$

Where, s is the total number of possible outcomes for an event (i.e. response to Mexican pheromone or response to standard pheromone), i is the total number of responses by an individual (i.e. 2-5 because a maximum of five tests were performed for each moth).

Results

U. S. Collected Moths A total of seventy five *A. nuxvorella* males from the US were tested. Of these 75 males, 17.3% (N= 13) never responded to either of the two pheromones. 82.7% (N= 62) of male *A. nuxvorella* responded at least once when tested (Figure 4.1). 61% (N=46) of males tested responded at least twice when tested with either of the pheromones used.

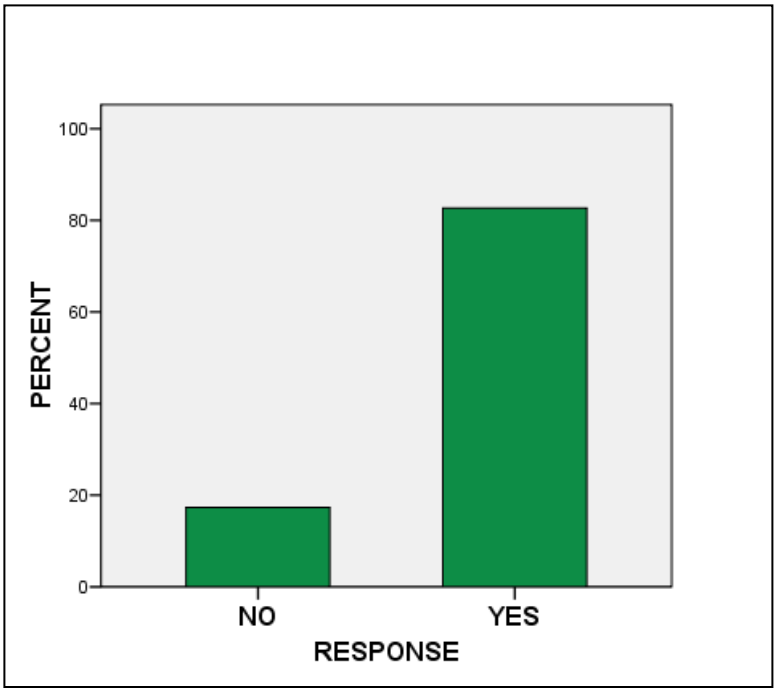

Fig. 4.1 Response rate for US collected male *A. nuxvorella*. 82.7% of individuals responded to one of the pheromones at least once, while 17.3% of individuals never responded

The first choice of each individual collected in the US was recorded and for those individuals that did respond at least once, no significant differences in first response were found (Figure 4.2; χ^2= 0.258, p>0.05. *df*=1).

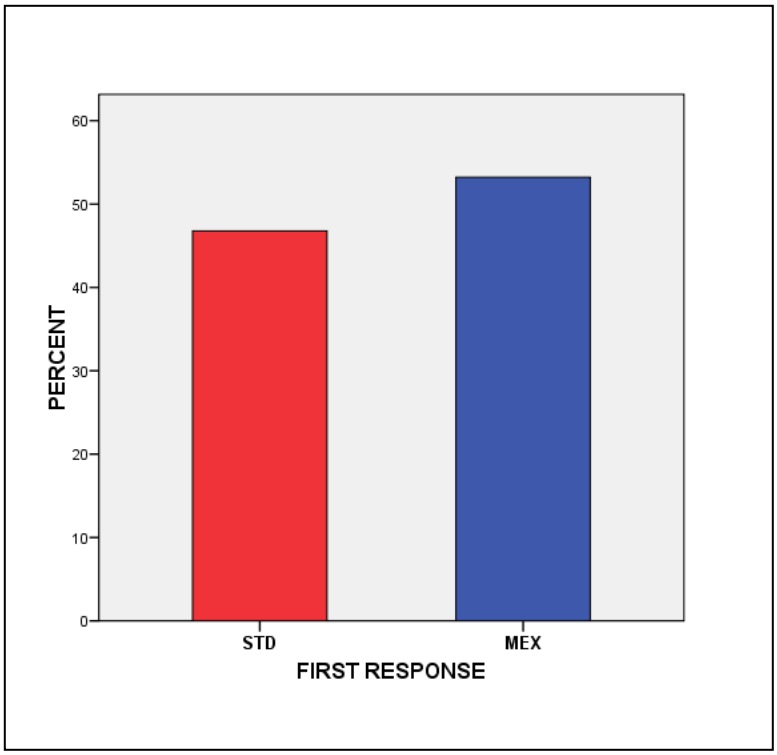

Fig. 4.2 First choice for US collected male *A. nuxvorella*. There were no significant differences found in the first response of males to the two tested pheromone blends. 53.2% of individuals chose the Mexican pheromone, while 46.8% of individuals chose the standard pheromone (χ^2= 0.258, p> 0.05. *df*=1)

Consistency of response of *A. nuxvorella* to the two pheromones was tested in order to determine if *A. nuxvorella* males would always choose the same pheromone (i.e. Mexican or standard) or if individuals would respond to different pheromones during

different testing periods. The results show that the number of male moths in the US showing the three potential behaviors we characterized (i.e., only response to standard, only response to Mexican and mixed response) were not different from what would have been expected by chance. Thus, no preference to consistently respond to any of the pheromones was found (Table 4.1, Figure 4.3; χ^2= 1.636, p> 0.05. *df*=2).

Table 4.1 χ^2 observed and expected value for each response behavior category of US collected *A. nuxvorella* males

Response Behavior	Observed	Expected
Mixed	27	30.28
Mexican	11	7.84
Standard	8	7.84

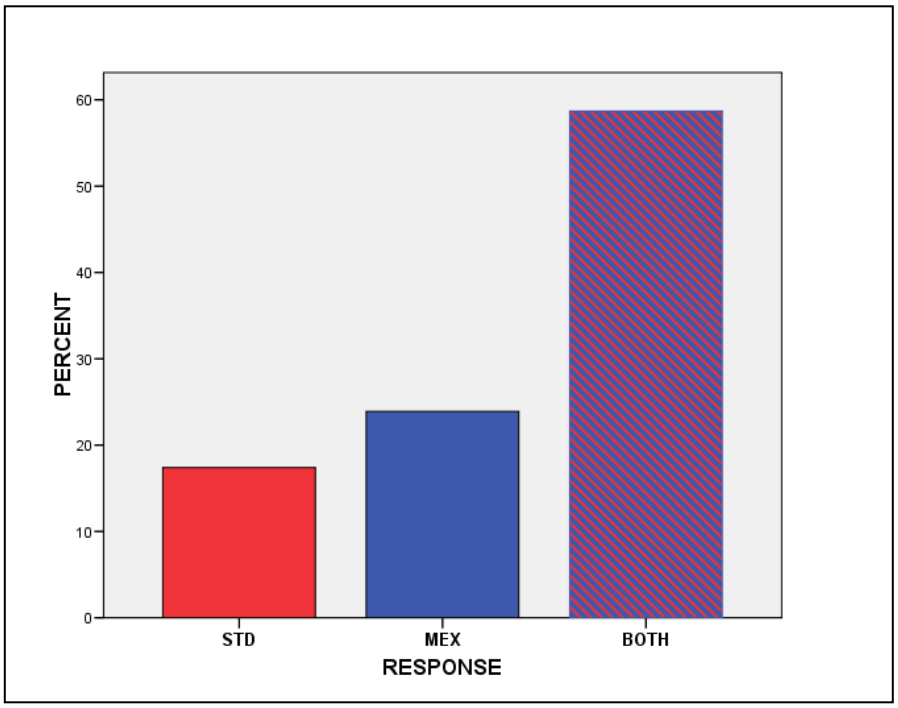

Fig. 4.3 Consistency of response of US collected male *A. nuxvorella* to the two pheromones. 17.39% of individuals tested consistently chose the standard pheromone, while 23.91% of individuals consistently chose the Mexican pheromone. 58.69% of the male *A. nuxvorella* chose both pheromones. There were equal proportions of male moths in the three mentioned categories (χ^2= 1.636, p> 0.05. *df*=2)

Mexican Collected Moths. Fifty nine *A. nuxvorella* males from Mexico were tested. Of these 59 males, 45.76% (N= 27) never responded to either of the two pheromones. 54.24% (N= 32) of male *A. nuxvorella* responded at least once when tested (Figure 4.4). 20.33% (N=12) of males tested responded at least twice when tested with either of the pheromones used.

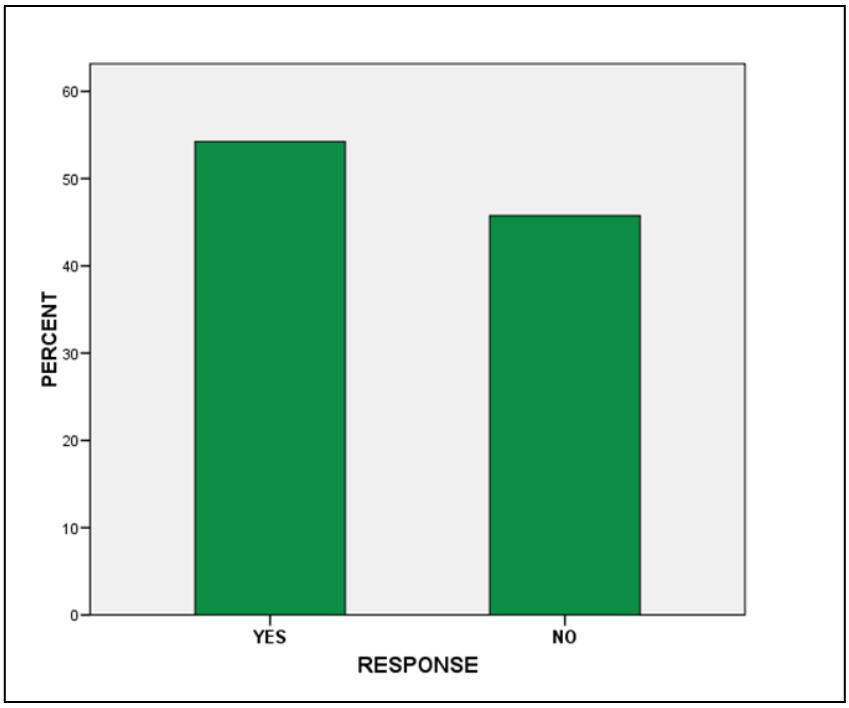

Fig. 4.4 Response rate for Mexican collected male *A. nuxvorella*. 54.24% of individuals responded to one of the pheromones at least once, while 45.76% of individuals never responded

The first choice of each Mexican collected individual was recorded and for those individuals that did respond at least once, no significant differences in first response were found (Figure 4.5; χ^2= 0.125, p> 0.05. *df*=1).

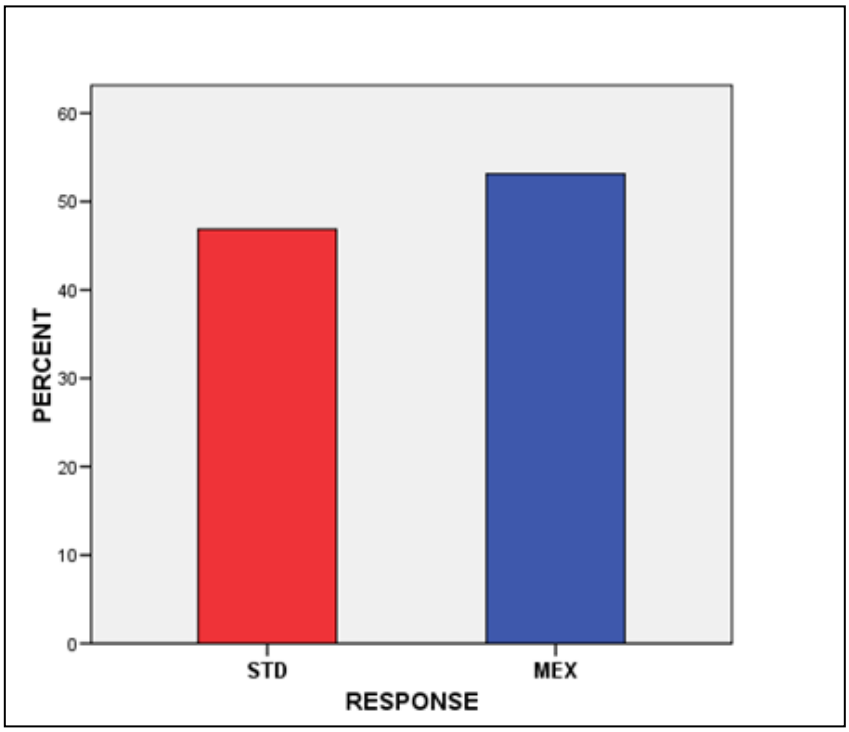

Fig. 4.5 First choice for Mexican collected male *A. nuxvorella*. There were no significant differences found in the first response of males to the two tested pheromone blends. 53.12% of individuals chose the Mexican pheromone, while 46.88% of individuals chose the standard pheromone (χ^2= 0.125, p> 0.05. *df*=1)

The results of the consistency of response test show that Mexican collected moths only exhibited two of the three potential behaviors. (i.e., only response to Mexican and mixed response). The results were not different from what would have been expected by chance. Thus, no preference to consistently respond to any of the pheromones was found (Table 4.2, Figure 4.6; Multinomial Exact Test = 1.7, p> 0.05. *df*=2).

Table 4.2 Multinomial exact test observed and expected value for each response behavior category of *A. nuxvorella* males collected in Mexico

Response Behavior	Observed	Expected
Mixed	9	7.25
Mexican	3	2.37
Standard	0	2.37

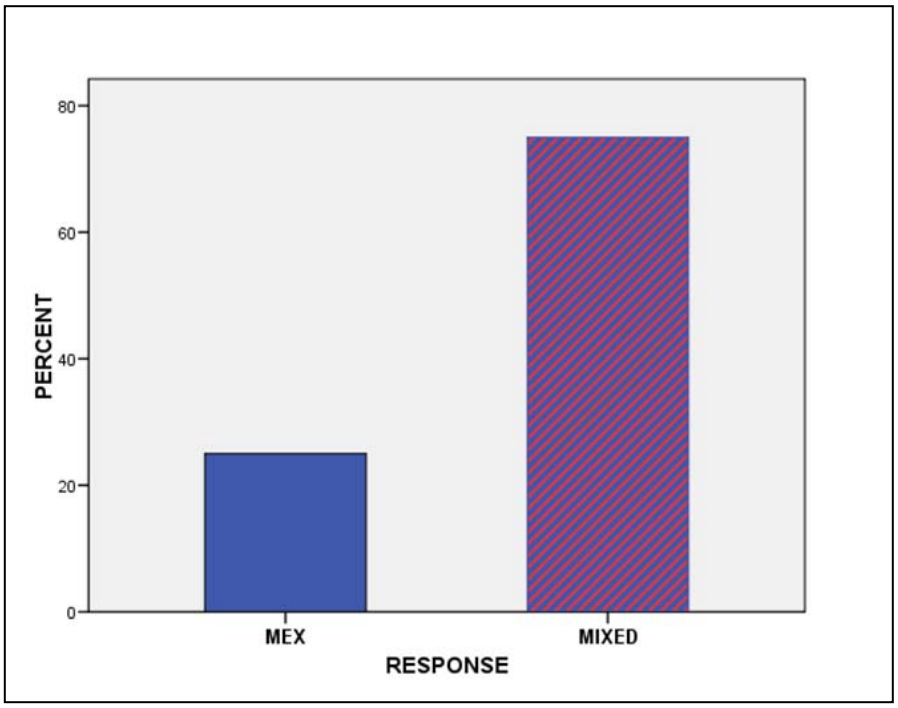

Fig. 4.6 Consistency of response for Mexican collected *A. nuxvorella* to the two pheromones. 0.0% of individuals tested consistently chose the standard pheromone, while 25% of individuals consistently chose the Mexican pheromone. 75% of the male *A. nuxvorella* chose both pheromones. (Multinomial Exact Test = 1.7, p> 0.05. *df*=2)

Discussion

The results of olfactometric testing suggest that male *A. nuxvorella* in the US and Mexico show no preference for either of the two pheromones tested. The results of the first choice made by *A. nuxvorella* males supports the hypothesis that within the US *A. nuxvorella* populations there are individuals capable of recognizing and responding to both the Mexican and standard pheromones. If male *A. nuxvorella* are in fact attracted equally to both pheromones, it is not surprising that the ratio of first choice among all individuals is relatively equal. These results of first response for Mexican collected moths, however, do not support the hypothesis that Mexican collected *A. nuxvorella* will not respond to the standard pheromone because almost half of those males tested initially chose the standard pheromone.

The initial hypothesis that male *A. nuxvorella* in the US are capable of recognizing and responding to both pheromones is further supported by the results of the consistency of response test. This is interesting because it supports the third assumption outlined in the asymmetric tracking hypothesis which states that male moths will be most sensitive to the most common pheromone present within a population, but male response should be wide enough to recognize potential mates of the same species, even if the calling female belongs to a different pheromone race (Phelan, 1992). Most US collected male *A. nuxvorella* (58.69%) exhibited this widened response and chose both of the pheromones during different testing periods. The results of this study are further supported by earlier findings that deny the existence of pheromone strain formation in *A. nuxvorella* in the US (Chapter II). If male *A. nuxvorella* have preferential response to a

pheromone (which the results of this study indicate that they do not), this could lead to assortative mating and divergent selection in nature. The lack of genetic differences between the Mexican and standard pherotypes provides further evidence that males in the US can and do respond to both pheromones in nature.

The consistency of response of Mexican collected *A. nuxvorella* show that some males will respond to the standard pheromone (i.e., within the mixed response behavior). However, it is interesting that no Mexican collected males consistently responded to the standard pheromone. The response of Mexican collected *A. nuxvorella* in this laboratory setting contradict the field results where no males in Mexico respond to the standard pheromone (Harris et al., 2008). This suggests that the laboratory environment is missing crucial elements that are present in the natural environment. It is possible that the standard (aldehyde only) pheromone degrades faster when left in environments with higher ambient temperatures such as those exhibited in Sonora, Mexico, rendering it undetectable by male *A. nuxvorella* (Table 4.3). Alternatively, *Diatraea saccharalis* F. (Lepidoptera: Crambidae) produces the same sex pheromone as the standard *A. nuxvorella* pheromone (i.e., (9*E*, 11Z)-hexadecadienal (9*E*, 11Z-16:Ald)) (Santangelo et al. 2002). It is possible that male *A. nuxvorella* can detect olfactory cues given off by *D. saccharalis* and avoid the standard pheromone when those cues are present, but are attracted to the standard pheromone in the absence of those cues. Olfactory cues produced by *D. saccharalis* have been shown to be a factor in attracting parasitoids of this insect (Setamou et al., 2002).

Table 4.3 Historic monthly average high temperatures for Hermosillo, Sonora, Mexico and College Station, Texas, USA during months of *A. nuxvorella* activity

MONTH	HERMOSILLO	COLLEGE STATION
APRIL	30.56	24.44
MAY	35.56	27.78
JUNE	37.78	31.67
JULY	37.78	34.40
AUGUST	37.22	36.67
SEPTEMBER	36.11	33.89
OCTOBER	32.22	31.11

Temperatures reported in ˚C

The results of this study offer support to the asymmetric tracking hypothesis. The data explicitly show that some individual male *A. nuxvorella* have a widened response window that reflects the variability in pheromone production of females. Male moths with widened response to pheromones experience higher reproductive potential due to their ability to recognize a greater number of conspecific females (McElfresh and Millar, 2001). This ability to recognize a larger portion of the females within a population may reduce competition with other males. Few direct examples of this ability to recognize females with differing pheromones exist in the literature. Liu and Haynes (1994) showed that *T. ni* males from what they refer to as mutant sex pheromone producing lab colonies preferred females that produced normal sex pheromones, but after 49 generations the males from mutant colonies displayed a widening response that included both pheromones and were responding to normal and mutant pheromones with equal frequency. This indicates that over time, when an aberrant pheromone blend is

introduced into a population, a widened response to females can develop within the males of a population.

Roelofs et al. (2002) presented another example that supports the asymmetric tracking hypothesis. This study showed that a widened response to sex pheromones is exhibited between *O. nubilalis* and its congener *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae). These two species are closely related and it was reported that a small percentage of males of these two species showed cross attraction when tested in a wind tunnel (Roelofs et al., 2002; Linn et al., 2003; Linn et al., 2006). The cause of this cross attraction is hypothesized to be due to a pseudogene (i.e. a gene that has lost protein coding ability and is homologous to a functional gene) that resurfaced as an activated Δ14 desaturase gene (Roelofs et al., 2002; Baker, 2002). Desaturases are enzymes involved in pheromone production. These enzymes are able to remove two hydrogen atoms in order to create a double bond in the fatty acyl chain of some pheromone molecules (Baker, 2002). It was suggested that the cross attraction exhibited between *O. nubilalis* and *O. furnacalis* offer direct evidence that sudden changes in pheromone chemistry are more likely to be the cause of shifts in pheromone production than gradual changes over time and that if this is the case, there is an increased possibility that "pheromone resistance" (i.e. a shift in chemical communication due to long term exposure to pheromones) can occur within a population (Roelofs et al., 2002).

Pheromone resistance is a real concern for IPM (Carde and Minks, 1995; Evenden and Haynes, 2001; Mochizukii, 2008) and could likely take place in an agricultural setting where pheromone based population control is being implemented.

Pheromone based control methods usually consist of mass release of synthetic pheromone to disrupt mating and/or setting traps baited with synthetic pheromone with the intention of killing the searching individual (Silverstein, 1981; Yamanaka, 2006). The continual exposure to sex pheromones has been shown to be sufficient pressure to cause shifts in pheromone production by female *T. ni* (Evenden and Haynes, 2001). Because *A. nuxvorella* males have shown that they are perhaps capable of widened response to pheromones, pheromone resistance is a phenomenon that pecan producers should be aware of as it could cause a shift in the pheromone production of *A. nuxvorella.*

The ability of some male *A. nuxvorella* to recognize and respond to the two available sex pheromones is important because it shows that some *A. nuxvorella* males show no preference to either of the two available pheromones. The presented study offers insight into the evolution of mate finding in the Lepidoptera via sex pheromones by supporting the asymmetric tracking hypothesis. The pecan nut casebearer, *A. nuxvorella*, offers a novel system in which to further study the evolution of new pheromones within a species. Future studies need to be completed in order to determine the mechanisms that drive the existence of the two pherotypes of *A. nuxvorella* in the US and why males from Mexico are responsive to the standard pheromone in the laboratory but not in the field.

CHAPTER V

RELATIVE ABUNDANCE AND FLIGHT PHENOLOGY OF TWO PHEROTYPES OF *Acrobasis nuxvorella* NEUNZIG (LEPIDOPTERA: PYRALIDAE)

Introduction

Since the discovery of the standard and Mexican pheromones produced by *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae) in 1994 and 2003, respectively, synthetic pheromones have been an important aid in the management of this key pest of pecan (Millar et al., 1998; Stevenson et al., 2003; Harris et al., 2008). Pheromone traps are utilized to monitor adult *A. nuxvorella* activity and when combined with other available integrated pest management (IPM) tactics, allow producers to more accurately time pesticide applications (Harris et al., 1997; Stevenson et al., 2003).

Currently, decision-making tools for improving the timing of pesticide applications include a degree day prediction model to predict the timing of infestation by *A. nuxvorella* (Ring et al., 1983), a sequential sampling plan to determine if the damage to pecan by *A. nuxvorella* is above economic threshold (Ring et al., 1983), manual inspection of pecan to determine the stage of infestation by *A. nuxvorella*, and monitoring for *A. nuxvorella* with pheromone traps (Harris et al., 1997).

The current IPM strategic plan for controlling *A. nuxvorella* has been helpful in timing of needed pesticide applications, allowing for more efficient chemical control. *A. nuxvorella* are most susceptible to pesticides as first instar larvae before entering the

nutlet to feed and it is crucial that chemical treatments are made before the larvae enter the protection of the nutlet. If applied at the optimal time, a single pesticide application will effectively control infestations of first generation *A. nuxvorella* larvae (Millar et al., 1996). The current IPM strategic plan has significantly reduced the amount of pesticides used from the amount that were applied when "calendar sprays" were previously implemented for control of *A. nuxvorella* (Harris et al., 1998). The reduction of pesticide use is also beneficial to pecan IPM as a whole because reduced pesticide use increases the chances that other foliar pests, such as aphids and mites, can be effectively controlled by natural enemies during later parts of the growing season (Harris et al., 1998).

For commercial pecan production there are several pesticides available to control *A. nuxvorella*, including pyrethroids, carbaryl, chlorpyrifos, endosulfan, azinophosmethyl, malathion, and phosmet (Knutson and Ree, 2004; Knutson and Ree, 2005). Pyrethroids and carbaryl are known to result in secondary infestations of aphids and mites and should be used with caution (Knutson and Ree, 2005). The entomopathogenic bacterium, *Bacillus thuringiensis* (*B.t.*) has been labeled for control of *A. nuxvorella* (Sundfram et al., 1997), but the effectiveness of *B.t.* at controlling *A. nuxvorella* is undocumented (Knutson and Ree, 2004; Sundfram et al., 1997). Additionally, there are over 25 known parasitoids associated with *A. nuxvorella* (Neunzig, 1972), but none are known to provide effective control when released in large numbers (Knutson and Ree, 2004).

The future IPM strategic plan for *A. nuxvorella* is population control through pheromone based technologies such as mate disruption or trap-and-kill. *A. nuxvorella* is

an excellent candidate for this type of control because male *A. nuxvorella* exhibit protandry (Roelofs, 1970), and the damage threshold for pecan nuts is relatively high at 10% (Ring, 1989). Pheromone based technologies have been successful at controlling lepidopteran pests such as *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) (Brooks et al., 1979; Baker, 1990), *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Cardé et al., 1977; Barnes et al., 1992), *Synanthedon scitula* Harris (Lepidoptera: Seiidae) (Leskey et al., 2006) and *Prays oleae* Bernard (Lepidoptera: Yponomeutidae) (Hegazi et al., 2009). In many cases pheromone based control has been shown to be as efficient as conventional pesticides (Cardé and Minks, 1995) and they do not have the same harmful environmental effects that accompany most pesticides.

One factor that could hinder the success of a pheromone based control method is the existence of two or more pherotypes. It is currently unknown whether the two *A. nuxvorella* pherotypes also exhibit differing phenologies that could have a negative affect on pheromone based control. Examples of lepidopteran species that exhibit polymorphic pheromone production and phenological differences include *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) in which there are: a bivoltine Z pheromone race (utilizing a 97:3 Z/E11-14:OAc pheromone blend), a bivoltine E pheromone race (utilizing a 1:99 Z/E11-14:Oac pheromone blend) and a univoltine E pheromone race (utilizing a 1:99 Z/E11-14:Oac pheromone blend) all existing in sympatry at many locations (Roelofs et al., 1985). Pashley et al. (1992) reported that the two host strains (later found to produce different pheromone blends by Groot et al. (2008)) of *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) showed differing temporal

activity throughout the year. The “corn strain” occurs in greatest numbers in the spring and mid summer, while activity of the “rice strain” increases in mid summer and fall.

In order to maximize the efficiency of the current IPM strategic plan in place to control *A. nuxvorella* and to successfully implement control methods based on pheromone technology, it is important to understand the relationship between the two pherotypes of *A. nuxvorella*. The main objective of this study was to determine the relative abundance of the two pherotypes and to verify that phenological differences that could hinder the control of *A. nuxvorella* with pheromone based technology do not exist between the pherotypes at each of 9 study sites selected from the geographic distribution of *A. nuxvorella*.

Methods and Materials

Data Collection In order to determine the relative abundance of each of the two pherotypes of *A. nuxvorella*, peer-cooperators and producer-cooperators deployed pheromone baited sticky traps (Trece Inc., Adair, OK) into pecan orchards and recorded daily counts of the number of *A. nuxvorella* captured at each of 9 study sites distributed across 4 states (i.e., Florida, Georgia, Oklahoma and Texas).

Researchers working throughout pecan growing regions in the US and Mexico randomly deployed 6 Trece Pherocon III™ sticky traps baited with lures containing the synthetic standard pheromone and 6 traps baited with lures containing the synthetic Mexican pheromone. Producer-cooperators who participated in this study deployed 3 traps baited with the standard pheromone and 3 traps baited with the Mexican pheromone.

The standard pheromone lures used for this study consisted of 10.7 mm gray butyl rubber caps impregnated with 100 µg of the synthetic *A. nuxvorella* standard pheromone (i.e. (9*E*,11*Z*)-hexadecadienal (9*E*, 11Z-16:Ald)) (Millar et al., 1996). The Mexican pheromone lure consisted of 10.7 mm red butyl rubber caps impregnated with 100 µg of the Mexican pheromone construct (i.e. (9*E*, 11Z)-hexadecadien-1-yl acetate (9*E*, 11Z-16:Ac): (9*E*,11*Z*-16:Ald)) (Harris et al., 2008). Pheromone lures were individually placed in Pherocon III™ Delta traps. Three traps at a location have been shown to be sufficient to characterize flight phenology of standard pherotype *A. nuxvorella* (Stevenson et al., 2003).

Pheromone baited sticky traps were placed into randomly selected pecan trees. Traps were placed near the tip of nut bearing branches, about 2 meters above the ground, maintaining a minimum distance of about 50 meters between traps (Stevenson et al., 2003). Counts of *A. nuxvorella* males captured in each trap were taken at least twice a week during the *A. nuxvorella* flight period. Counts were recorded on a Microsoft Excel (Microsoft Corporation, Redmond, WA) spreadsheet provided to each participant via email. Data were then sent back to Texas A&M University in College Station, Texas, where they were compiled and analyzed.

Data Analyses The average number of moths captured per pheromone trap type (i.e. Mexican or standard pheromone lure used) was calculated for each location and year. Data was then analyzed using SPSS 15.0 statistical software (SPSS, 2006). A split plot design analysis of variance (ANOVA) was used to determine if the number of male *A.*

nuxvorella captured varied due to the type of pheromone lure used and/or, due to the location of the collection site. Tukey's test was used to perform mean pairwise comparisons. The Bonferroni correction for multiple comparisons was applied to the Tukey's post hoc analyses.

Emergence curves were then constructed in order to assess whether or not flight phenology differed for either of the two pherotypes at each location. Peak trap capture, (i.e. 50% cumulative trap capture (Bartles et al., 1999)) was used to directly assess the emergence of male *A. nuxvorella* from the pupal stage. Trap catch is a sufficient indicator of emergence in male *A. nuxvorella* because males are responsive to pheromone lures the first scotophase after emergence from the pupal stage (personal observation).

Results

The results of the split plot ANOVA are presented in table 5.1, and show a significant main effect of pheromone ($F= 4.611$; $p= 0.033$). Significantly more male *A. nuxvorella* were captured using the standard pheromone lure than the Mexican pheromone lure (Figure 5.1). State was also a significant main effect ($F= 13.649$; $p= 0.005$). Post hoc Tukey's tests were conducted in order to compare mean moth catches in the different states considered. The significance of the Tukey's test comparisons was assessed after applying the Bonferroni correction. In Texas, significantly more *A. nuxvorella* were captured using the Mexican pheromone than in any of the other 3 states. In contrast, the number of male *A. nuxvorella* captured using the Mexican pheromone in Florida,

Oklahoma and Georgia were not significantly different from each other (Figure 5.2). The number of *A. nuxvorella* captured using the standard pheromone lure was significantly larger in Texas than in Florida or Georgia, but not significantly larger than the number captured in Oklahoma. The number of moths captured in Oklahoma using the standard pheromone blend was not significantly different from the number at any other location (Figure 5.3).

Table 5.1 Results of split plot ANOVA

Interaction	*df*	Mean Square	F	Significance
YEAR	2	472.159	0.376	0.697
STATE	3	16504.403	13.649	0.005**
PHEROMONE	1	7030.401	4.611	0.033**
PHEROMONE* STATE	3	134.290	0.088	0.967
STATE* YEAR	5	1189.314	0.780	0.565

Year – Year that data were collected (2007, 2008, or 2009)
Pheromone – Pheromone lure used (Mexican or standard)
State – State from which moths were collected (Texas, Georgia, Florida, or Oklahoma)
** Significantly different at 0.05 level

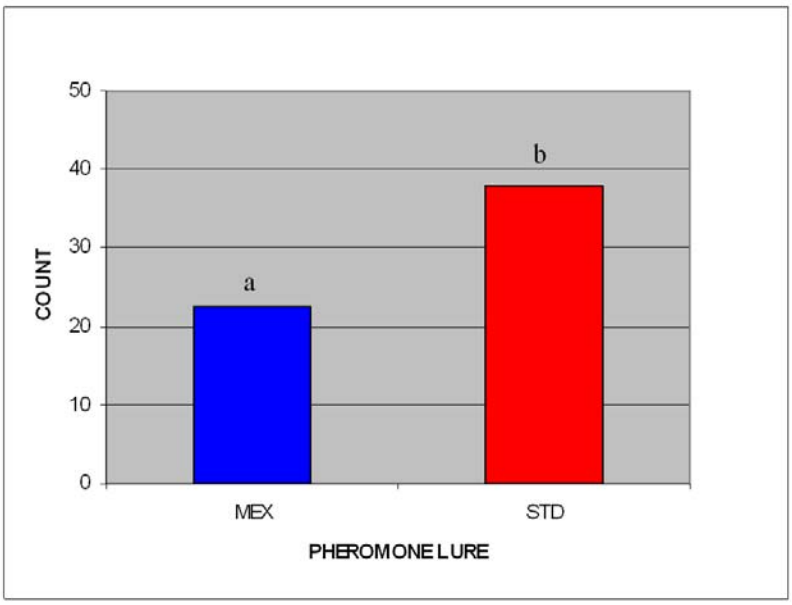

Fig. 5.1 Average number of moths captured using the Mexican (MEX) and standard (STD) pheromone lures. The number of *A. nuxvorella* captured using the standard pheromone lure was significantly larger than the number captured using the Mexican pheromone lure (ANOVA, p=0.033)

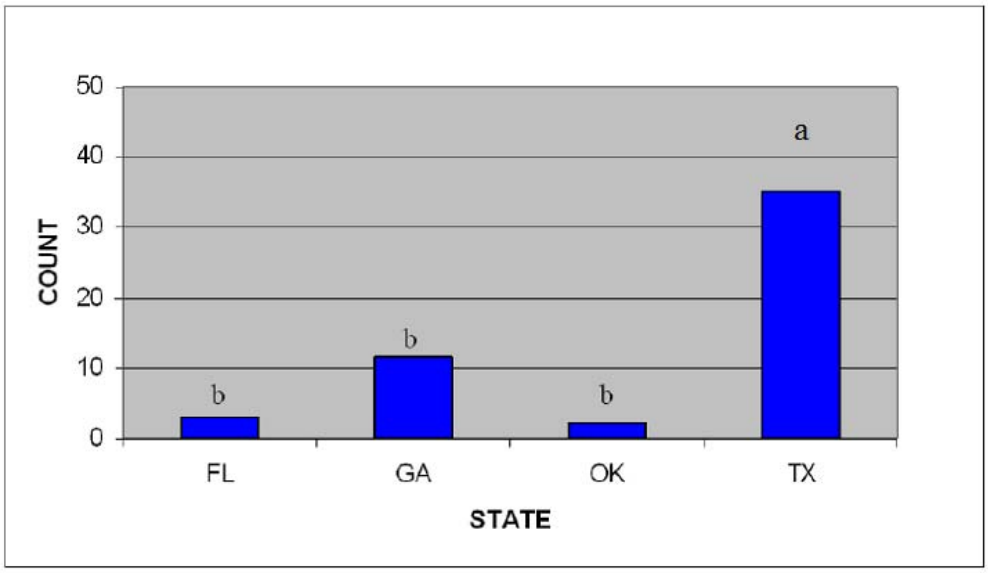

Fig. 5.2 Average number of male *A. nuxvorella* captured with the Mexican pheromone lure. Categories labeled with the same letter are not significantly different from each other at the 0.05 level (Tukey's post hoc test, after Bonferroni post hoc correction)

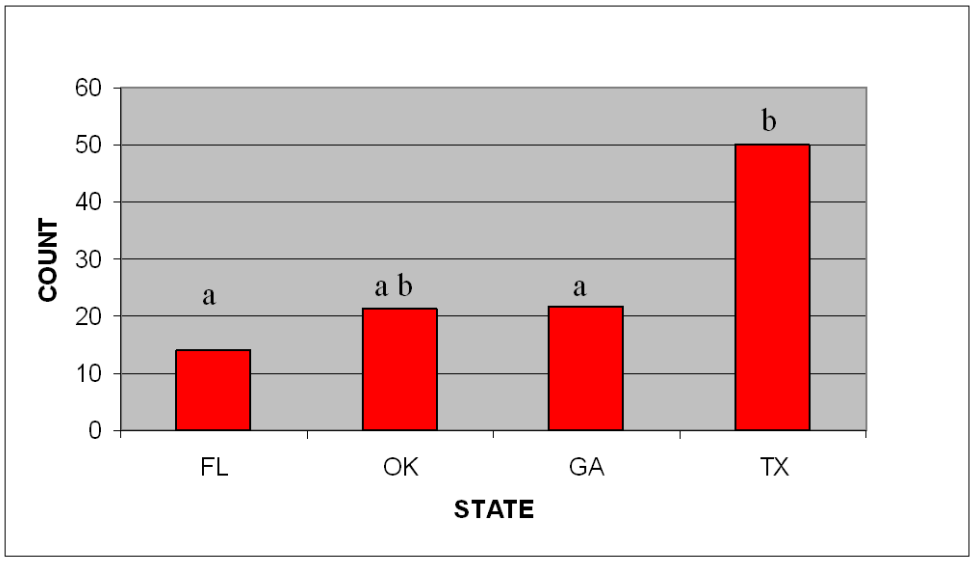

Fig. 5.3 Average number of male *A. nuxvorella* captured with the standard pheromone lure. Categories labeled with the same letter are not significantly different from each other at the 0.05 level (Tukey's post hoc test, after Bonferroni post hoc correction)

The cumulative percentage of emergence for each location for 2007 (Figure 5.4a), 2008 (Figure 5.4b), and 2009 (Figure 5.4c) are presented. There was no yearly or state by state pattern in which pherotype reached peak emergence first. The differences in the peak emergence at each location ranged from 0 to ~5 days. Most of the emergence curves have a sigmoid shape. Two, however (Oklahoma 2009 and Florida 2009), have odd shapes, and this is due to a small number of observations at these locations.

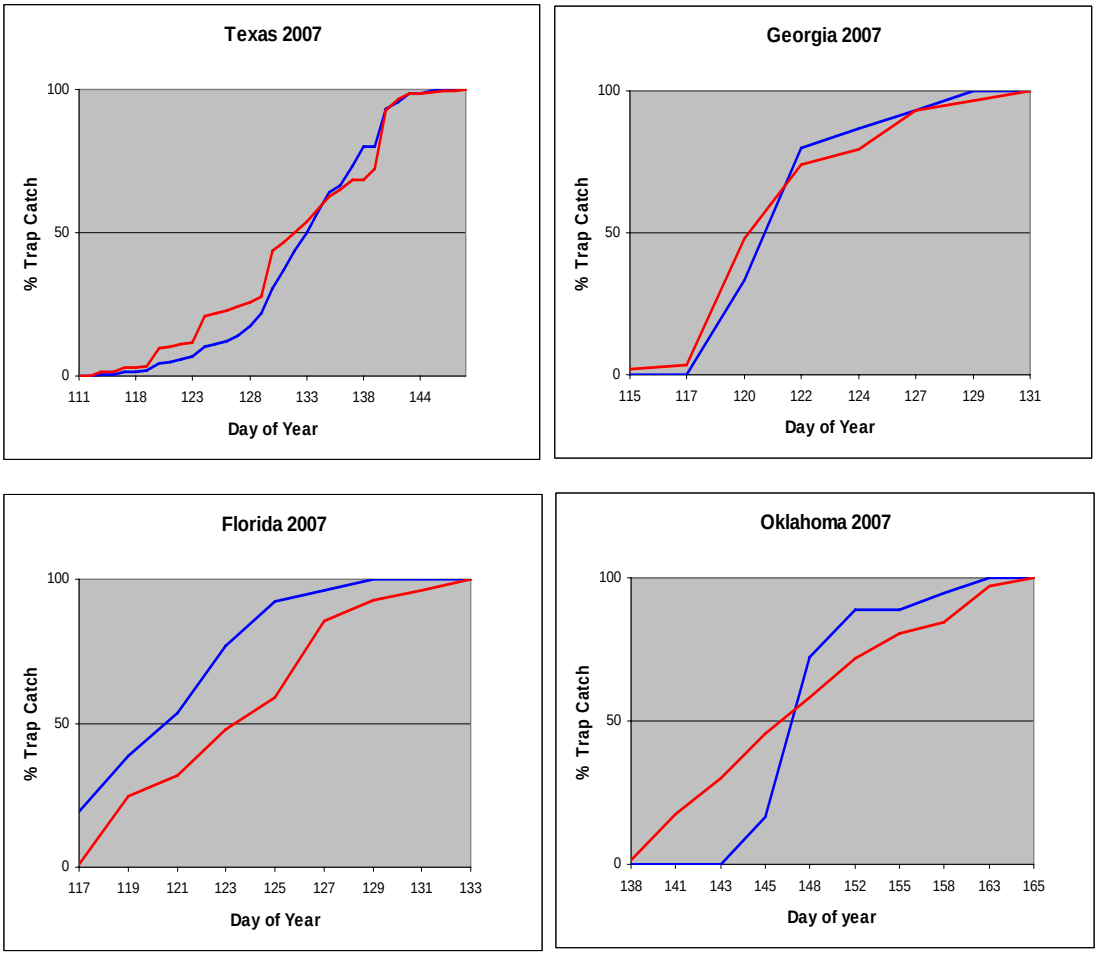

Fig. 5.4a Cumulative percentage trap catch of *A. nuxvorella* using Mexican (blue line) and standard (red line) pheromone lures for Texas, Georgia, Florida and Oklahoma in 2007.

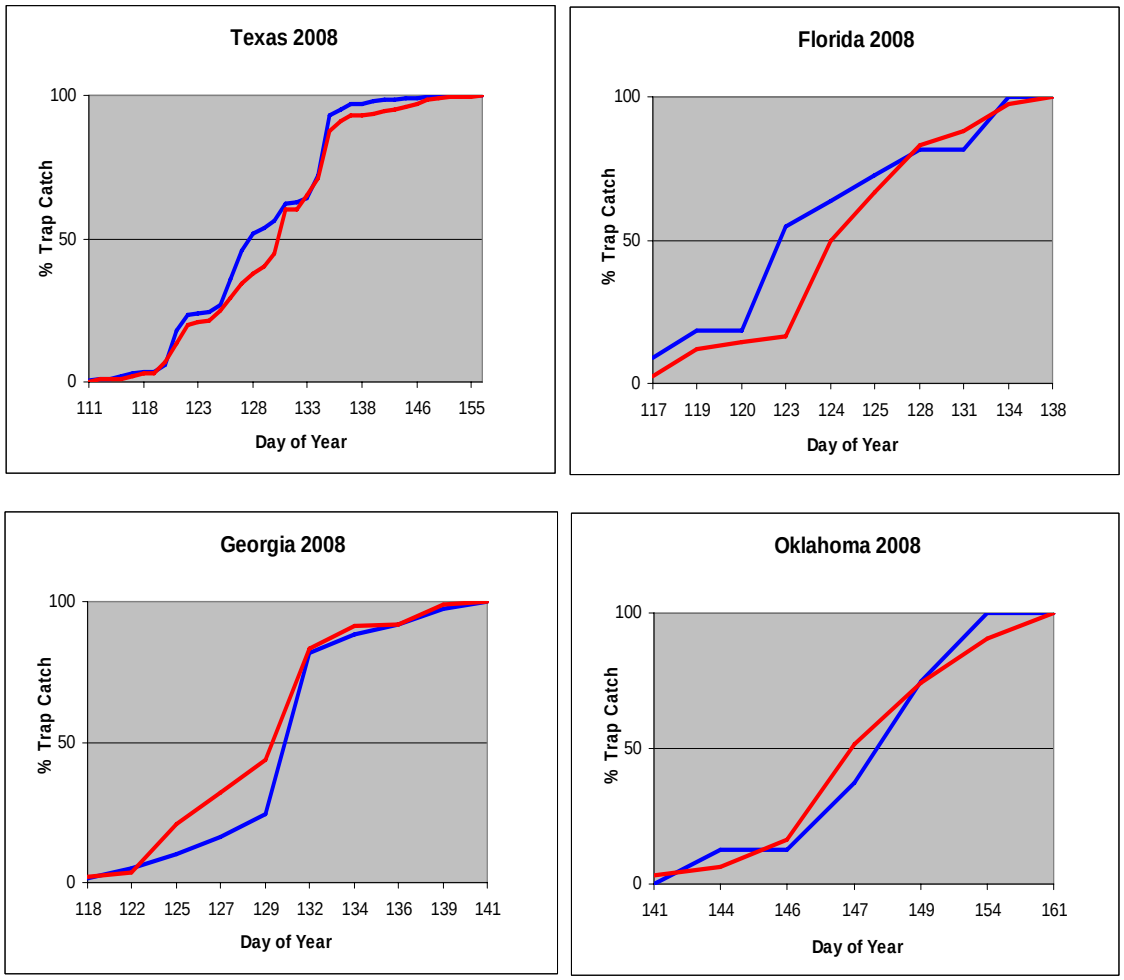

Fig. 5.4b Cumulative percentage trap catch of *A. nuxvorella* using Mexican (blue line) and standard (red line) pheromone lures for Texas, Georgia, Florida and Oklahoma in 2008.

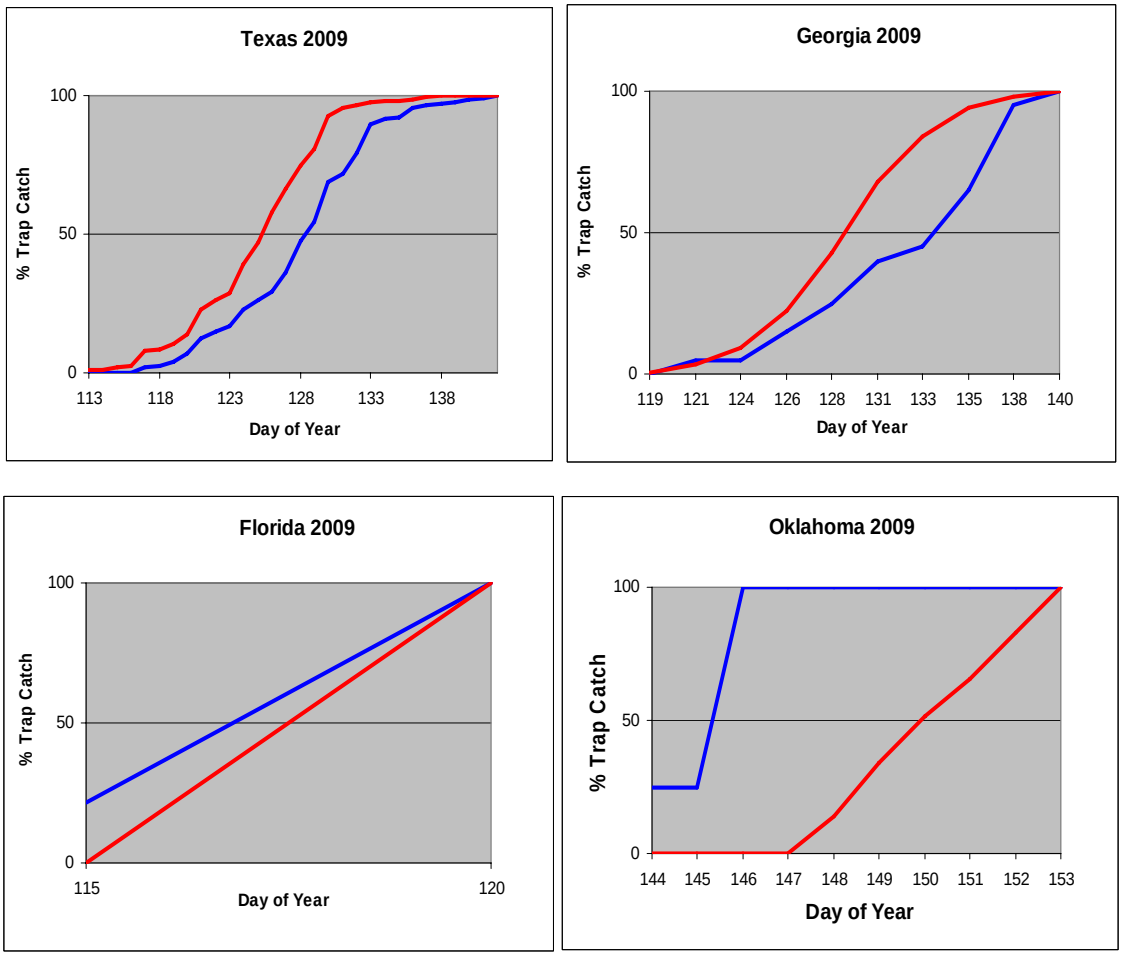

Fig. 5.4c Cumulative percentage trap catch of *A. nuxvorella* using Mexican (blue line) and standard (red line) pheromone lures for Texas, Georgia, Florida and Oklahoma in 2009.

Discussion

The results of ANOVA show that there are significantly more male *A. nuxvorella* captured with the standard pheromone than with the Mexican pheromone at each location sampled in the US. The reason for this discrepancy is unknown, but may be explainable by the asymmetric tracking hypothesis. The third parameter of this

hypothesis (see Chapter IV) states that in populations that show polymorphic pheromone production, males will be most responsive to the most common pheromone present, but will still have the capabilities to respond to all pheromones produced by conspecific females (Phelan, 1992). The large numbers of males captured in the standard pheromone baited traps indicate that the standard pheromone is the most common blend produced by females.

There was also a significant difference between the numbers of male *A. nuxvorella* captured in each state. The discrepancies in the numbers of moths captured in each state may be influenced by the acreage of pecan grown in that state. The trend of the average number of moths captured per trap at each collection site shown in figures 5.2 and 5.3 correspond to the total estimated pecan acreage for each of those states. Wood (2001) reported (based on data derived from the 1997 United States Agricultural Census) that Texas pecan production takes place on 167,841 acres, Georgia pecan production takes place on 131,872 acres, Oklahoma pecan production takes place on 83,837 acres and Florida pecan production takes place on 9,533 acres. Increased pecan acreage leads to increased habitat for *A. nuxvorella* which could allow for larger population numbers.

No obvious differences in pattern were observed in the flight phenology of the two *A. nuxvorella* pherotypes considered (i.e., standard and Mexican pherotypes) at any of the collection sites during the 3 years this study was conducted. This is important for the control of *A. nuxvorella* because the first generation, which is the most destructive, is most effectively controlled when pesticide applications are made before the first instar

larvae enter the protection of the developing pecan nutlet to feed. If peak emergence of *A. nuxvorella* is the same for the two pherotypes, this is an indication that oviposition by females, larval growth and pupation probably follow a similar pattern. If this is true, then first generation larvae of both pherotypes can effectively be controlled with a single, well timed pesticide application. The lack of differences in flight phenology is beneficial to the implementation of pheromone based control methods. Male *A. nuxvorella* typically emerge a couple of days before females. Because males of both pherotypes appear to emerge at the same time, the chance that males will be captured in pheromone traps and killed before calling females emerge is increased.

The results of this study show that significant differences in the number of male *A. nuxvorella* captured using each pheromone blend exist, but it is still important that producers continue to use traps baited with both pheromone blends in pecan orchards to accurately assess population densities. Similarities in flight phenology between the two pherotypes make *A. nuxvorella* a candidate for population control using pheromone based technology. Before this type of control can be implemented more needs to be known about *A. nuxvorella* females including the age at which females begin calling for mates. If females begin calling a few days after emergence, as opposed to the first scotophase after eclosion, this will further increase the chance that synthetic pheromones can be used to effectively control populations of this key pest of pecans.

CHAPTER VI

CONCLUSIONS

Summary of Presented Research

Two sex pheromones have been described for *Acrobasis nuxvorella* Neunzig (Lepidoptera: Pyralidae), the most damaging pest of pecans (Millar et al., 1996; Harris et al., 2008). These two pheromones have been implemented by producers as an important tool in monitoring the activity of this pest (Stevenson et al., 2003; Harris et al., 2008). Based on the discovery of the two *A. nuxvorella* pheromone blends, it was hypothesized that there were two pheromone strains of this species (Harris et al., 2008). The results of the studies presented in Chapters II, IV, and V work together to verify that the suspected pheromone strains within *A. nuxvorella* do not exist, while Chapter III offers information on the genetic structure of *A. nuxvorella* across the geographic distribution of this pest.

Pheromones did not prove to be a reproductive isolating mechanism between pherotypes of *A. nuxvorella* when amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) were used to detect genetic differences (Chapter II). However, it is possible to have two pherotypes within the same species that are not reproductively isolated, and there are examples of this in other Lepidoptera (McElfresh and Millar, 2001; Liu and Haynes, 1994). The occurrence of more than one pheromone blend within *A. nuxvorella* without reproductive isolation can be explained by the asymmetric tracking hypothesis which states that male moths will be most sensitive to the most common pheromone blend present within a population, but that male response should be wide enough to recognize potential mates of the same species, even if the

calling female belongs to a different pheromone race (Phelan, 1992). When this hypothesis was tested on *A. nuxvorella* from the US and Mexico, it was determined that this is the case for this species (Chapter IV). Some male *A. nuxvorella* showed no preference to either of the two pheromones available for this species when tested in a y-tube olfactometer, supporting the idea that male *A. nuxvorella* are capable of recognizing and responding to both of the sex pheromones produced by females. The lack of phenological differences in the timing of emergence of the two pherotypes of *A. nuxvorella* further substantiate that there are not pheromone strains within this species (Chapter V). The coincidence of the emergence of the two pherotypes provides evidence that there are no temporal reproductive isolating mechanisms with *A. nuxvorella*.

On the other hand, the results of AFLP analyses indicate a high degree of genetic structure in *A. nuxvorella* across its geographic distribution (Chapter III). The most highly genetically differentiated populations occurred in areas where pecan is not native, but has been cultivated. These genetic differences within *A. nuxvorella* populations can be attributed to founder effects that resulted from the introduction of *A. nuxvorella* into new areas, the high degree of habitat fragmentation in areas where pecan is cultivated and thus pecan patches are less contiguous than pecan stands within the native range, and the relatively large distances between the introduced populations and the populations within the native range of pecan. The lack of genetic differentiation within the native range of pecan also showed some indication that long range dispersal of this insect is limited to areas where pecan is present contiguously and occurs with less fragmentation, as is the case in the native range of pecan.

The Existence of Two Pherotypes of *A. nuxvorella*

Further research is needed in order to explain the existence of the two characterized *A. nuxvorella* pherotypes. The asymmetric tracking hypothesis explains one avenue by which multiple pherotypes can exist within populations. This hypothesis states that the majority of males within a population will respond to the most common pheromone produced by conspecific females, but that males should also be able to track changes in female pheromone production (Phelan 1992). This results in the ability of males to be responsive enough to recognize females that produce alternative pheromones.

Although it is unknown how the two pheromones utilized by *A. nuxvorella* came to exist, it is likely that one of the blends originated as a mutant form of the other blend. This has been shown to be the cause of polymorphic pheromone production in other species such as *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) (Hunt and Haynes, 1990). Although it is currently unknown whether or not genetics play a factor in differential pheromone production in *A. nuxvorella*, it is highly likely because pheromone production and response has been shown to be controlled by one or a few genes in other lepidoptera (Löfstedt, 1993).

This thesis presents evidence that suggests that the factors that lead to differential pheromone production by females of this species do not lead to reproductively isolate the two *A. nuxvorella* pherotypes into distinct pheromone strains. Thus, it is possible that in Mexico, *A. nuxvorella* respond only to the Mexican pheromone because genes

associated with recognition of the Mexican pheromone were selected for while genes responsible for the response to the standard pheromone were selected against.

Furthermore, the absence of the standard pheromone in Mexico may be attributed to the fact that the sugarcane borer, *Diatraea saccharalis* F. (Lepidoptera: Crambidae) also produces the same sex pheromone (9*E*, 11Z)-hexadecadienal (9*E*, 11Z-16:Ald) (Santangelo et al., 2002). It is possible that *A. nuxvorella* in Mexico are unresponsive to this pheromone as a mechanism to avoid cross attraction between the two species.

Future Directions

The long term goal of this research is to provide the insight needed to successfully achieve population control of *A. nuxvorella* through pheromone based control methods. *A. nuxvorella* has already proven to be a good candidate for this type of control because a synthetic sex pheromone that is competitive with the female produced pheromone is available, this species exhibits protandry and pecan has a relatively high damage threshold (Ring et al., 1983). One concern that should not be overlooked is the possibility of *A. nuxvorella* males developing pheromone resistance. Male produced pheromones should also be considered. In some species of the Phycitinae, the sub-family to which *A. nuxvorella* belongs, males have been shown to produce sex pheromones (Phelan, 1997). The production of male pheromones may allow for short range attraction of females and hinder the success of pheromone based control (Carde and Minks, 1995). Before implementing pheromone based population control, these factors should be examined.

The response of Mexican collected *A. nuxvorella* males to the standard pheromone in laboratory environments and not in natural environments should be explored further. It is possible that differences in temperature cause the synthetic standard pheromone to degrade faster in the field, or it is possible that the lack of olfactory cues from *Diatraea saccharalis* F. (Lepidoptera: Crambidae) causes Mexican collected males to respond to the standard pheromone in the laboratory.

The underlying mechanisms that drive the existence of the pherotypes within *A. nuxvorella* are not well understood and are in need of further examination. Possible avenues of exploration include: biosynthetic pathways in female pheromone production, the genetic control of female pheromone production and male response, and the role that pheromone neuron receptors play in male olfaction.

Finally, the range of *A. nuxvorella* has expanded into most North American pecan growing regions east of the Rocky Mountains (Fu Castillo et al., 2005; Stevenson et al., 2003). In Chapter III it was shown that the Sierra Madre Occidental Mountain Range is a geographic barrier between *A. nuxvorella* in Hermosillo (Sonora, Mexico) and the central US. Future population genetic studies should be conducted to include more locations within the distribution of *A. nuxvorella* to determine if the Appalachian Mountain Range is also a geographic barrier for *A. nuxvorella* populations. This in turn will offer insight into whether or not the Rocky Mountains will serve as an adequate geographic barrier preventing the westward expansion of *A. nuxvorella* into areas where this pest is currently not established. If the Rocky Mountains prove to be an ineffective

barrier to the movement of *A. nuxvorella*, intensive monitoring and quarantine methods may need to be implemented to prevent the further expansion of this devastating pest.

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